

CHIMERIC WEST NILE/DENGUE VIRUSES

CROSS REFERENCE TO RELATED APPLICATION

This claims the benefit of U.S. Provisional Application No. 61/049,342, filed
5 April 30, 2008, which is incorporated herein in its entirety.

FIELD

The disclosure relates to chimeric flaviviruses, particularly chimeric West Nile virus/Dengue virus constructs. Further, it relates to methods of using these
10 chimeras in diagnosis of flavivirus infection and assessing candidate Dengue virus vaccine efficacy.

BACKGROUND

Dengue virus (DENV) is the most important arboviral cause of morbidity
15 and mortality throughout the world. There are currently 2.5 billion people living in dengue endemic regions with roughly 100 million annual cases of dengue fever and hundreds of thousands of cases of dengue hemorrhagic fever and dengue shock syndrome (Gubler, *Clin. Microbiol. Rev.* 11:480-496, 1998). No vaccines are currently commercially available against any of the four DENV serotypes (DENV 1-
20 4) largely because vaccine production is hampered by the fact that neutralizing antibodies to one serotype do not effectively neutralize the remaining DENV serotypes (Halstead and O'Rourke, *J. Exp. Med.* 146:201-217, 1977). In fact, low levels of these antibodies may actually increase the risk for more severe disease during secondary infection due to a phenomenon known as antibody mediated
25 enhancement, which occurs when antibodies against one DENV serotype bind in a non-neutralizing manner to DENV particles of another serotype. This binding allows increased infection of Fc receptor-bearing cells, such as macrophages, which can change the infection profile of the virus or cause a release of chemokines
30 O'Rourke, *J. Exp. Med.* 146:201-217, 1977).

West Nile virus (WNV) is a member of the Japanese encephalitis serocomplex in the genus *Flavivirus*, family *Flaviviridae*. Until the mid-1990s,

WNV caused sporadic outbreaks of illness in Africa, the Middle East, and Western Asia. However, since 1996, WN encephalitis has been reported more frequently in Europe, the Middle East, northern and western Africa, and Russia. WNV emerged in the western hemisphere in 1999 and has become the leading cause of arboviral encephalitis in humans and equines in North America. There are two lineages of WNV. Lineage 1, of which the NY99 strain is a member, is the more virulent strain and is the predominant strain infecting humans and horses (Jordan *et al.*, *Viral Immunol.* 13:435-446, 2000). There is currently no approved vaccine for WNV to protect at-risk human populations from WN illness.

SUMMARY

Disclosed herein are chimeric flaviviruses including non-coding regions, non-structural proteins, and a C protein from WNV, and at least a portion of a prM protein and E protein from DENV. In some embodiments, the chimera includes a first nucleic acid molecule including a 5' non-coding region, a nucleic acid encoding a C protein and non-structural proteins, and a 3' non-coding region from a West Nile virus and a second nucleic acid molecule operably linked to the first nucleic acid molecule, encoding at least a portion of a prM protein and E protein from a Dengue virus. In a particular example, the chimeric flavivirus includes nucleotide sequence(s) from DEN2 virus.

Also disclosed are chimeric flaviviruses including non-coding regions and non-structural proteins from WNV and at least a portion of a C protein, prM protein, and E protein from DENV. In some embodiments, the chimera includes a first nucleic acid molecule including a 5' non-coding region, a nucleic acid encoding non-structural proteins, and a 3' non-coding region from a WNV and a second nucleic acid molecule operably linked to the first nucleic acid molecule, encoding at least a portion of a C protein, a prM protein, and an E protein from a DENV.

In some examples, the chimeric flavivirus includes at least one nucleic acid or amino acid substitution which improves chimera characteristics (such as increased replication in cell culture or decreased infectivity or transmissibility in mosquitoes). In particular examples, the amino acid substitution is in the DENV prM protein, DENV E protein, WNV NS2A protein, or WNV NS4B protein. In

additional examples, the chimeric flavivirus includes at least one nucleotide substitution in the 5' or 3' non-coding region.

In further examples, the chimeric flavivirus includes at least one amino acid substitution in the DENV E protein, wherein the substituted E protein exhibits
5 measurably reduced antibody cross-reactivity.

Also disclosed herein are methods of using the chimeric flaviviruses in diagnosis of flavivirus infection. In a particular embodiment, the method includes detecting Dengue virus antibody in a sample, including contacting a sample from a subject with a chimeric flavivirus disclosed herein and detecting formation of an
10 antibody-virus complex. In some embodiments methods of use of the chimeric flavivirus to evaluate efficacy of candidate Dengue virus vaccines are disclosed. Also disclosed are methods of producing Dengue virus structural proteins utilizing the chimeric flaviviruses described herein.

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

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic diagram of a WN/DEN2 chimeric flavivirus. The
20 chimera contains the prM signal sequence (between the NS2B-3 protease and signalase cleavage sites, arrow and triangle, respectively), prM, and E proteins of the DEN2 16681 virus in the WN NY99 virus backbone. The enlarged sequence alignment shows the SacII restriction site (underlined) that was introduced in the cDNA clone to create the splice site to engineer the chimera. The mutations
25 introduced to create the SacII site do not change the DEN2 amino acid sequence.

Figure 2 shows the plaque phenotype of chimeric WN/DEN2 virus seed recovered from C6/36 cells. The amplified virus seed was plated on Vero cells to visualize plaques. Fig. 2A shows plaques formed by the chimeric WN/DEN2 virus on day 8 post-infection (p.i). Fig. 2B shows plaques formed by DEN2 16681 virus
30 on day 8 p.i.

Figure 3 shows the plaque phenotype of WN/DEN2 virus recovered from C6/36 cells or Vero cells on day 5 p.i. at the indicated passages. (A) wild type

WNV NY99 (LLC-MK2-1); (B) wild type DEN2 16681 (C6-1); (C) WN/DEN2 (C6-1); (D) WN/DEN2 (C6-1/V-2); (E) WN/DEN2 (V-3); (F) WN/DEN2 (V-10).

Figure 4 shows growth curves in Vero cells of the wild type WNV NY99 (wt WNV), wild type DEN2 16681 virus (wt DENV-2), and successive passages of WN/DEN2 seeds (WN/DEN2 C6-1 seed, C6-1/V-2 seed, V-3 seed, and V-10 seed). Each point represents an average titer from three flasks, with the error bars showing the highest and lowest titers at each time point. ♦, wild type WNV; ■, wild type DEN2; ▲, WN/DEN2; C6-1 seed; ×, WN/DEN2 C6-1/V-2 seed; *, WN/DEN2 V-3 seed; ●, WN/DEN2 V-10 seed.

Figure 5 shows growth curves in C6/36 cells of the wild type WNV NY99 (wt WNV), wild type DEN2 16681 virus (wt DENV-2), and successive passages of WN/DEN2 seeds (WN/DEN2 C6-1 seed, C6-1/V-2 seed, V-3 seed, and V-10 seed). ♦, wild type WNV; ■, wild type DEN2; ▲, WN/DEN2; C6-1 seed; ×, WN/DEN2 C6-1/V-2 seed; *, WN/DEN2 V-3 seed; ●, WN/DEN2 V-10 seed.

Figure 6 shows plaque phenotype of WN/DEN2 chimeras engineered to include E-203, NS2A49, and/or NS2A94 mutants. (A) WN/DEN2 C6-1 seed; (B) WN/DEN2 E-N203D V-1 seed; (C) WN/DEN2 NS2A-I49T/F94L V-1 seed; (D) WN/DEN2 N203D, NS2A-I49T/F94L V-1 seed.

Figure 7 shows the midgut infection rate of wild type WNV-NY99, wild type DENV-2 16681, and WN/DEN2 C6-1 seed in *Culex pipiens*, *Culex quinquefasciatus*, and *Aedes aegypti* mosquitoes. Open bars, DENV-2; shaded bars, WNV-NY99; solid bars, WN/D2 C6-1 seed.

Figure 8 is a schematic diagram of a WN/DEN chimeric flavivirus. The chimera contains the prM signal sequence from the indicated DEN virus (between the NS2B-3 protease and signalase cleavage sites, arrow and triangle, respectively), and prM and E proteins of the DEN virus in the WN NY99 virus backbone. The enlarged sequence alignment shows junction between the WNV C protein and the DENV prM signal sequence in the WN/DEN1, WN/DEN3, and WN/DEN4 chimeras.

SEQUENCE LISTING

Any nucleic acid and amino acid sequences listed herein or 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. §

- 5 1.822. In at least some cases, 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.

SEQ ID NOs: 1 and 2 show the nucleic acid and amino acid sequences, respectively, of a recombinant West Nile/Dengue-2 chimera WN/DEN2. The start and stop positions of the particular genes and proteins of the chimera are shown in Table 1.

SEQ ID NOs: 3 and 4 show the nucleic acid and amino acid sequences, respectively, of a recombinant West Nile/Dengue-1 chimera. The start and stop positions of the particular genes and proteins of this chimera are shown in Table 1.

15 SEQ ID NOs: 5 and 6 show the nucleic acid and amino acid sequences, respectively, of a recombinant West Nile/Dengue-3 chimera. The start and stop positions of the particular genes and proteins of the chimera are shown in Table 2.

SEQ ID NOs: 7 and 8 show the nucleic acid and amino acid sequences, respectively, of a recombinant West Nile/Dengue-4 chimera. The start and stop positions of the particular genes and proteins of this chimera are shown in Table 1.

Table 1. Start and stop positions of NCRs, structural proteins and nonstructural proteins in WN/DEN2, WN/DEN1, and WN/DEN4 chimeras

Region	Nucleotide start/stop position (SEQ ID NOs: 1, 3, and 7)	Amino acid start/stop position (SEQ ID NOs: 2, 4, and 8)
5' NCR	1-96	--
C	97-453	1-119
prM	454-951	120-285
M	727-951	211-285
E	952-2436	286-780
NS1	2437-3492	781-1132
NS2A	3493-4185	1133-1363
NS2B	4186-4578	1364-1494
NS3	4579-6435	1495-2113
NS4A	6436-6882	2114-2262
NS4B	6883-7647	2263-2517
NS5	7648-10362	2518-3422

Region	Nucleotide start/stop position (SEQ ID NOs: 1, 3, and 7)	Amino acid start/stop position (SEQ ID NOs: 2, 4, and 8)
3' NCR	10363-10996	--

Table 2. Start and stop positions of NCRs, structural proteins and nonstructural proteins in SEQ ID NOs: 5 and 6. (WN/DEN3 chimera)

Region	Nucleotide start/stop position (SEQ ID NO: 5)	Amino acid start/stop position (SEQ ID NO: 6)
5' NCR	1-96	--
C	97-453	1-119
prM	454-951	120-285
M	727-951	211-285
E	952-2430	286-778
NS1	2431-3486	779-1130
NS2A	3487-4179	1131-1361
NS2B	4180-4572	1362-1492
NS3	4573-6429	1493-2111
NS4A	6430-6876	2113-2260
NS4B	6877-7641	2261-2515
NS5	7642-10356	2516-3420
3' NCR	10357-10990	--

5 SEQ ID NOs: 9 and 10 show the nucleic acid and amino acid sequences, respectively, of the C protein/prM junction in a wild type DEN2 16681 virus.

 SEQ ID NOs: 11 and 12 show the nucleic acid and amino acid sequences, respectively, of the WN/DEN2 chimeric virus.

 SEQ ID NOs: 13 and 14 show the nucleic acid and amino acid sequences, respectively, of the C protein/prM junction in a wild type WN NY99 virus.

 SEQ ID NOs: 15 and 16 show the nucleic acid and amino acid sequences, respectively, of the WN/DEN1 chimeric virus.

 SEQ ID NOs: 17 and 18 show the nucleic acid and amino acid sequences, respectively, of the WN/DEN3 chimeric virus.

15 SEQ ID NOs: 19 and 20 show the nucleic acid and amino acid sequences, respectively, of the WN/DEN4 chimeric virus.

DETAILED DESCRIPTION

 Lack of an ideal DEN animal model is a major obstacle in vaccine and therapeutic development for DENV. Immunocompetent outbred mice do not

succumb to wild type DENV infection, so typical markers of protection, such as lethality and viremia, are not evident in mice after wild type DENV challenge. Transgenic and inbred mice have been used for DENV mouse models, but these animals are usually high cost, difficult to work with, and are not realistic for high-throughput or multiple dose experiments. Many animals are susceptible to WNV infection, and outbred mice, such as Swiss Webster and NIH Swiss, succumb to wild type WNV infection. Sickness, lethality, and viremia level have been successfully used as protection markers in WNV research using small animal models such as, mice, hamsters, and birds. Thus, chimeric WN/DEN viruses disclosed herein may be virulent and/or generate significant viremia in mice, therefore they can be used as the challenge dose to assess the efficacy of DENV candidate vaccines.

In addition, although both DENV and WNV are flaviviruses, DENV replicates much more slowly and to lower titers than WNV in cell cultures. This makes development of diagnostic viral antigen production and diagnostic tests for DENV more difficult than for WNV. The chimeric WN/DEN viruses described herein contain DENV antigenic structures on the surface of the virus particles while retaining certain WNV replication features (such as replication to high titer). The disclosed chimeras can thus be used as a DEN-like surrogate virus for testing DENV candidate vaccine efficacy and for development of faster or more effective DENV diagnostics.

I. Abbreviations

DEN: Dengue
DENV: Dengue virus
E: envelope glycoprotein
ELISA: enzyme-linked immunosorbent assay
HMAF: hyperimmune mouse ascitic fluid
IFA: immunofluorescence antibody assay
mAb: monoclonal antibody
MOI: multiplicity of infection
NCR: non-coding region
pfu: plaque forming unit
p.i.: post-infection
prM: premembrane protein
PRNT: plaque reduction neutralization test
VD₅₀: 50% virulent dose
WN: West Nile

WNV: West Nile virus

WN/DEN: West Nile/Dengue virus chimera

II. Terms

5 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
10 (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 invention, the following explanations of specific terms are provided:

Antibody: A protein (or protein complex) that includes one or more
15 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
20 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
25 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
30 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
5 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
10 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*
15 *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
20 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
25 (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
30 epitope recognized by the antibody paratope.

In one example, antibody binding affinity is measured by end-point titration in an Ag-ELISA assay. Antibody binding affinity is substantially lowered (or

measurably reduced) by the modification and/or substitution of one or more amino acids in the epitope recognized by the antibody paratope if the end-point titer of a specific antibody for the modified/substituted epitope differs by at least 4-fold, such as at least 10-fold, at least 100-fold or greater, as compared to the unaltered epitope.

5 **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
10 E protein.

Chimera: A molecule (*e.g.*, gene, transcript or protein) composed of parts that are of different origin (such as at least two nucleic acid or amino acid sequences) that, while typically unjoined in their native state, are joined or linked to form a single continuous molecule. A chimera may include nucleotide or amino
15 acid sequences 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).

20 A chimera may include a chimeric protein, for example a protein that is composed of amino acid sequences from more than one protein. A chimera may also include a chimeric nucleic acid sequence 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,
25 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 West Nile virus and a Dengue virus. In some examples, a chimeric flavivirus includes nucleic acid sequences encoding one or more proteins from a first flavivirus and nucleic acid
30 sequences encoding one or more proteins from a second flavivirus. In particular examples, a chimeric flavivirus is composed of a nucleotide sequence encoding the non-structural proteins and a C protein from a West Nile virus genome linked to a

nucleotide sequence encoding at least a portion of a prM protein and E protein from a Dengue virus genome (such as DEN1, DEN2, DEN3, or DEN4).

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 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 ability to replicate (for example as described in Example 2), by producing virus containing a variant protein and determining its neurovirulence or neuroinvasion properties (as described in Example 6), or by testing antibody cross-reactivity (as described in Example 10).

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 cross-reactive antibody: An antibody that recognizes (that is, specifically binds to) an epitope found on a peptide from more than one species of flavivirus. Flavivirus cross-reactive antibodies are classified as either complex cross-reactive or group cross-reactive antibodies. Complex cross-reactive antibodies recognize epitopes shared by all viruses within a complex, such as the JE virus complex or the DEN virus complex. Group cross-reactive antibodies recognize epitopes shared by all members of the genus *Flavivirus*.

Antibody cross-reactivity is further refined within the sub-complex and sub-group cross-reactive categories. Sub-complex cross-reactive antibodies recognize epitopes shared by most, but not all, members of a particular flavivirus complex (e.g., DEN-1, -2, and -3, but not DEN-4), while sub-group cross-reactive antibodies
5 recognize epitopes shared by flaviviruses from several complexes, but not all members of the flavivirus group (e.g., all members of the DEN virus and JE virus complexes, but not all members of the tick-borne virus complex). Specific, non-limiting examples of flavivirus cross-reactive antibodies include the group cross-reactive mAbs 4G2 and 6B6C-1, the sub-group cross-reactive mAb 1B7-5, and the
10 sub-complex cross-reactive mAb 10A1D-2. (see, e.g., Roehrig *et al.*, *Virology* 246:317-28, 1998; Crill and Chang, *J. Virol.* 78:13975-13986, 2004).

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.
15 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,
20 1993). NS2 is cleaved into NS2A and NS2B, with the function of NS2A remaining unknown. 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
25 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
30 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).

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.

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 biological components in the cell of the organism in which the component naturally 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.

10 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.

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).

Recombinant nucleic acid: A nucleic acid molecule 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 that have been altered solely by addition, substitution, or deletion of a portion of a natural nucleic acid molecule.

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.

Orthologs (equivalent to proteins of other species) of proteins are in some instances characterized by possession of greater than 75% sequence identity counted over the full-length alignment with the amino acid sequence of specific protein using ALIGN set to default parameters. Proteins with even greater similarity to a
5 reference sequence will show increasing percentage identities when assessed by this method, such as at least 80%, at least 85%, at least 90%, at least 92%, at least 95%, at least 98%, or at least 99% sequence identity. In addition, sequence identity can be compared over the full length of one or both binding domains of the disclosed fusion proteins.

10 When significantly less than the entire sequence is being compared for sequence identity, homologous sequences will typically possess at least 80% sequence identity over short windows of 10-20, and may possess sequence identities of at least 85%, at least 90%, at least 95%, 96%, 97%, 98%, or at least 99% depending on their similarity to the reference sequence. Sequence identity over such
15 short windows can be determined using LFASTA; methods are described at the NCBI website. One of skill in the art will appreciate that these sequence identity ranges are provided for guidance only; it is entirely possible that strongly significant homologs could be obtained that fall outside of the ranges provided. Similar homology concepts apply for nucleic acids as are described for protein. An
20 alternative indication that two nucleic acid molecules are closely related is that the two molecules hybridize to each other under stringent conditions.

Nucleic acid sequences that do not show a high degree of identity may nevertheless encode similar amino acid sequences, due to the degeneracy of the genetic code. It is understood that changes in nucleic acid sequence can be made
25 using this degeneracy to produce multiple nucleic acid sequences that each encode substantially the same protein.

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).

30 **Transformed:** A "transformed" cell is a cell into which has been introduced a nucleic acid molecule 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, amelioration, or treatment of infectious or other types of disease. The immunogenic material may include attenuated or killed microorganisms (such as bacteria or viruses), or antigenic proteins, peptides or DNA derived from them. An attenuated vaccine 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. A killed vaccine is a previously virulent microorganism that has been killed with chemicals or heat, but elicits antibodies against the virulent microorganism. Vaccines may elicit both prophylactic (preventative) 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.

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. Similarly, the word "or" is intended to include "and" unless the context clearly indicates otherwise. 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. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety for all purposes. All GenBank Accession Nos. mentioned herein are incorporated by reference in their entirety. 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. 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. WN/DEN Chimeric Viruses

5 Disclosed herein are chimeric flaviviruses that include non-coding regions, non-structural proteins, and a C protein from WNV, and at least a portion of a prM protein and E protein from DENV. In some embodiments, the chimera includes a first nucleic acid molecule including a 5' non-coding region, a nucleic acid encoding a C protein and non-structural proteins, and a 3' non-coding region from a West Nile
10 virus genome and a second nucleic acid molecule operably linked to the first nucleic acid molecule, encoding at least a portion of a prM protein and E protein from a Dengue virus genome. In particular examples, the nucleic acid molecules encoding the prM and E proteins of the WNV genome are replaced with molecules having the corresponding sequences from the DENV genome. In some examples, the prM
15 signal sequence of the WNV C protein is also replaced with the prM signal sequence of the corresponding DENV genome.

Also disclosed are chimeric flaviviruses including non-coding regions and non-structural proteins from WNV and at least a portion of a C protein, prM protein, and E protein from DENV. In some embodiments, the chimera includes a first
20 nucleic acid molecule including a 5' non-coding region, a nucleic acid encoding non-structural proteins, and a 3' non-coding region from a WNV genome and a second nucleic acid molecule operably linked to the first nucleic acid molecule, encoding at least a portion of a C protein, a prM protein, and an E protein from a DENV genome. In a particular example, the nucleic acid encoding the C, prM, and
25 E proteins of the WNV genome are replaced with molecules having the corresponding sequences from the DENV genome.

In some examples disclosed herein, the WNV genome used in the chimera is derived from a particular WNV strain, such as NY99 or KEN-3829. Additional WNV strains are known in the art (see, e.g., Ebel *et al. Emerg. Infect. Dis.* 7:650-
30 653, 2001; American Type Culture Collection (ATCC) catalog numbers VR-82, VR-1267, VR-1507, VR-1510). In particular examples, the WNV genome is WN/IC-P991 (such as GenBank Accession No. AF196835 (incorporated by reference as

included in GenBank on April 27, 2009) or with mutations as described in Kinney *et al.*, *J. Gen. Virol.* 87:3611-3622, 2006).

WNV genome sequences are publicly available. For example, GenBank Accession Nos.: AF196835, AY278441, AF202541, AF404754, AF260967, 5 AY660002, AF481864, AY268133, AF404757, AY268132, AF260969, AF317203, AY262283, AY490240, AF260968, AY603654, D00246, M12294, EU068667, AY765264, and AY277251 disclose WNV genomic nucleic acid sequences, all of which are incorporated by reference as included in GenBank on April 27, 2009. In further examples, the WNV genome, or the non-coding regions, non-structural 10 proteins, and/or C protein of the WNV genome are at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to a publicly available WNV genome sequence.

In the disclosed flavivirus chimeras, the DENV genome is from a Dengue 1 (DEN1), Dengue 2 (DEN2), Dengue 3 (DEN3), or Dengue 4 (DEN4) virus. In some 15 examples, the DENV genome portion of the disclosed chimeras includes sequences from a single DENV genome, while in other examples, the DENV genome portion includes sequences from two or more DENV genomes. The DENV genome may be a wild type strain or an attenuated (or vaccine) strain. In some examples, the DENV genome is DEN2 (for example, wild type DEN2 16681 strain or attenuated DEN-2 20 PDK-53 strain), DEN1 (for example, wild type DEN1 16007 strain or attenuated DEN1 PDK-13 strain), DEN3 (for example, wild type DEN3 16562 strain or attenuated DEN3 PGMK-30/FRhL-3) or DEN4 (for example, wild type DEN4 1036 or attenuated DEN4 PDK-48). Additional DENV strains are known in the art (see *e.g.*, U.S. Pat. Nos. 5,939,254 and 6,793,488). In particular examples, the DENV 25 genome is a wild type (non-attenuated) strain, for example DEN2 16681 (such as GenBank Accession No. U87411, incorporated by reference as included in GenBank on April 27, 2009).

DENV sequences are publicly available. For example GenBank Accession Nos.: NC_001477, AF180817, and U88536 disclose DEN1 nucleic acid sequences; 30 NC_001474 and U87411 disclose DEN2 nucleic acid sequences; NC_001475, AY099336, and AF317645 disclose DEN3 nucleic acid sequences; and NC_002640 and AF326825 disclose DEN4 nucleic acid sequences, all of which are incorporated

by reference as included in GenBank on April 27, 2009. In additional examples, the DENV genome (or the prM and/or E protein from the DENV genome) are at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to a publicly available DENV sequence.

5 In a particular embodiment, the chimeric flavivirus includes a nucleic acid molecule having a sequence including the 5' and 3' non-coding regions of the virus and encoding the non-structural proteins and C protein from a WN NY99 virus genome, operably linked to a nucleic acid molecule having a sequence encoding the prM signal sequence, the prM protein and the E protein from DEN2 16681 virus
10 genome. In one example, the chimeric flavivirus is a WN/DEN2 chimera having the nucleic acid and amino acid sequence shown in SEQ ID NOs: 1 and 2, respectively. In additional examples, the disclosed chimeric virus has nucleic acid and amino acid sequences at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical or more to the sequences disclosed in SEQ ID NOs: 1 and 2.

15 In other embodiments, the chimeric flaviviruses disclosed herein include a first nucleic acid molecule including the 5' and 3' non-coding regions and encoding the non-structural proteins and C protein from a WN NY99 virus genome operably linked to a second nucleic acid molecule encoding the prM and E proteins from a DEN1, DEN3, or DEN4 virus genome. In some examples, the second nucleic acid
20 molecule encodes the prM signal sequence from a DEN1, DEN3, or DEN4 virus genome. Particular examples of WN/DEN1 (SEQ ID NOs: 3 and 4), WN/DEN3 (SEQ ID NOs: 5 and 6) and WN/DEN4 (SEQ ID NOs: 7 and 8) chimeras are disclosed herein. In additional examples, the disclosed chimeric viruses are at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical or more to the
25 sequences disclosed in SEQ ID NOs: 3-8.

 The disclosed chimeric flaviviruses 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
30 preferably 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.

Preferably, cells are 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 can be adapted to growth in low serum or serum-free

5 medium. 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). Suitable cell lines can be obtained from the American Type Culture Collection (ATCC), Manassas, VA.

10 In some examples, the disclosed chimeric WN/DEN viruses replicate in cell culture more rapidly than DEN viruses. For example, plaques formed by WN/DEN chimeric viruses may form on cell cultures (such as C6/36 or Vero cells) sooner than DEN viruses (such as at least one day, two days, three days, four days, or five days post-infection sooner). In other examples, WN/DEN chimeric viruses may form
15 larger plaques than DEN viruses. For example, plaques formed by chimeric WN/DEN viruses disclosed herein may form plaques that are at least 25% larger to about 10 times larger than DEN viruses (such as at least 50% larger, two-fold, three-fold, four-fold, five-fold, or up to 10-fold larger).

20 **IV. WN/DEN Chimeras and Variants Thereof**

The disclosure also provides flavivirus chimeras having one or more nucleic acid or amino acid substitution, insertion, deletion, or combination thereof, such that the resulting chimera has improved characteristics. In some examples, the improved characteristic of the chimera including one or more substitution, insertion, and/or
25 deletion includes, but is not limited to, increased virus titer, increased replication rate, increased plaque size, or increased stability in cell culture compared to a wild type virus. In additional examples, the improved characteristic of the chimera comprising one or more substitution, insertion, and/or deletion, includes increased infectivity or virulence in a subject (such as mice or non-human primates) or
30 decreased infectivity or transmissibility in mosquitoes as compared to a wild type virus.

Manipulation of the nucleotide sequence of the disclosed chimeric flaviviruses using 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
20	Asn	Gln, His
	Asp	Glu
	Cys	Ser
	Gln	Asn
	Glu	Asp
25	His	Asn; Gln
	Ile	Leu, Val
	Leu	Ile; Val
	Lys	Arg; Gln; Glu
	Met	Leu; Ile
30	Phe	Met; Leu; Tyr
	Ser	Thr
	Thr	Ser
	Trp	Tyr
	Tyr	Trp; Phe
35	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; (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,
5 is substituted for (or by) an electronegative residue, for example, glutamyl or aspartyl; 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.

In addition to targeted mutagenesis to produce variants of the disclosed WN/DENV chimeras, naturally occurring mutations may accrue upon passage in
10 cell culture that result in variants, some with desirable characteristics. Nucleic acid and amino acid substitutions, insertions, 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 WN/DENV chimera variants that have improved
15 characteristics (such as replication to high titer or production of uniform large plaques in cells). 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
20 chimeric viruses with improved characteristics (Huang *et al.*, *J. Virol.* 77:11436-11447, 2003; Huang *et al.*, *J. Virol.* 12:7300-7310, 2005).

In some embodiments, the chimeric flavivirus encodes a polypeptide that includes one or more amino acid substitution (or insertion or deletion) of one or more residues of the Dengue virus prM or E protein, such that the chimera has
25 improved characteristics. In other examples, the chimeric flavivirus encodes a polypeptide that includes one or more amino acid substitution (or insertion or deletion) of one or more residues of a WNV non-structural and/or C protein, such that the resulting chimera has improved characteristics. In additional examples, the chimeric flavivirus includes one or more nucleic acid substitution, insertion, or
30 deletion in the WNV 5' and/or 3' non-coding region, such that the chimera has improved characteristics.

Examples of a chimera encoding at least one substitution that improves chimeric virus characteristic are those encoding a polypeptide having one or more amino acid substitution in a DENV E protein. In particular embodiments, the substitution includes, but is not limited to, at least one substitution at DENV E protein amino acid position 64, 122, 186, or 203 of the DEN2 E protein, or a combination of two or more thereof. It is to be understood that substitutions at the equivalent E protein amino acid positions in DEN1, DEN3, or DEN4 are also contemplated. Furthermore, substitutions at the equivalent position are contemplated even in situations where the wild type amino acid is different from the amino acid in the wild type DEN2 (for example, DEN2 E protein includes Lys at position 64, while the equivalent amino acid in DEN4 E protein is Ser64). In some examples, the amino acid substitution alters a positively charged residue (such as Lys, Arg, or His; for example, Lys64 or Lys122) to a non-charged residue (for example, Met, Ser, Thr, Gly, Ala, Val, Leu, Ile, or Val) or to a negatively charged residue (for example, Asp or Glu). The particular substitution (expressed as in DEN2) may include K64M, K64S, K64T, K122L, K122I, K122T, and/or K122E. In other examples, the amino acid substitution alters a polar residue (such as Ser or Thr; for example, Ser186 of DEN2) to a negatively charged residue (for example, Asp or Glu) or a hydrophobic residue (such as phenylalanine). In further examples, the amino acid substitution includes a substitution at amino acid position 203 of the E protein (expressed as in DEN2), for example N203D. In particular examples, the disclosed chimeric flavivirus encodes a DEN2 E protein including K122I, S186F, N203D, or a combination of two or more thereof.

In some embodiments, the disclosed chimeric flavivirus encodes at least one amino acid substitution in the DENV prM protein that improves virus characteristics. In one example, the substitution includes a substitution at amino acid position 149 of the DEN2 prM protein. It is to be understood that substitutions at the equivalent prM protein amino acid positions in DEN1, DEN3, or DEN4 are also contemplated. Furthermore, substitutions at the equivalent position are contemplated even in situations where the wild type amino acid is different from the amino acid in the wild type DEN2 (for example, DEN2 prM protein includes Phe at position 149, while the equivalent amino acid is Thr in DEN1 and DEN3 and is Ile in DEN4). In a

particular example, the amino acid substitution alters Phe149 of DEN2 prM to a noncharged amino acid (for example, Met, Thr, Gly, or Ile), such as an amino acid residue that is found at the equivalent position in the Japanese encephalitis complex (for example, WNV, Japanese encephalitis virus, St. Louis encephalitis virus, or
5 Murray Valley encephalitis virus).

In additional embodiments, the disclosed chimeric flavivirus may encode at least one amino acid substitution in each of the DENV prM and E proteins. In other examples, the chimeric flavivirus may encode two or more amino acid substitutions in the DEN prM protein or the E protein. For example, the chimeric flavivirus may
10 include at least one amino acid substitution in the DEN E protein (such as Lys64, Lys122, Ser186, and/or Asn203) and at least one amino acid substitution in the DEN prM protein (such as Phe149).

In further examples, the disclosed chimeric flavivirus encodes at least one amino acid substitution, insertion, or deletion in at least one non-structural protein
15 (for example, NS1, NS2A, NS2B, NS3, NS4A, NS4B, or NS5) or C protein of the WNV that improves virus characteristics. For example, the chimeric flavivirus may encode one or more amino acid substitutions in non-structural protein NS2A that may increase virus titer, replication rate, or plaque size, or may stabilize growth of the disclosed chimeras in cell culture. In some examples, the amino acid substitution
20 may include substitutions at one or more of Val23 of NS2A (such as V23M or V23C), Ile49 of NS2A (such as I49T), and Phe94 (such as F94L). Other variant chimeras may encode one or more substitutions in non-structural protein NS1, NS3, and/or NS4A. These variants may alter virus characteristics, for example increasing temperature sensitivity or decreasing infectivity in mosquitoes. In particular
25 examples, the substitution may include Gly53 of NS1 (for example G53D). In other examples the substitution may include amino acid positions 249 and/or 251 of NS3 (such as P249T, P249H, E251V, or E251Q). In still further examples, the flavivirus chimera may encode one or more substitution in non-structural protein NS4B (such as Thr241 of NS4B, for example T241I).

30 In some embodiments, the disclosed chimeras may include at least one nucleotide substitution, insertion, or deletion in the 5' and/or 3' non-coding region of the WNV backbone, such that the substitution, insertion, or deletion improves virus

characteristics such as replication rate, virus titer, plaque size, stability in cell culture, or infectivity in mammals or mosquitoes. In one example, the chimera includes insertion of a microRNA (miRNA, such as miR-14 (Mead and Tu, *BMC Genomics* 9:244, 2008)) in the 5' or 3' non-coding region to decrease virus
5 replication in mosquitoes. In other examples, the nucleic acid substitution, insertion, and/or deletion may decrease virus replication in mosquitoes or mice.

In additional examples, the chimeras include a combination of two or more nucleic acid substitutions in the non-coding regions, in the nucleic acid sequences encoding the C, prM, E, or non-structural proteins, or any combination thereof. For
10 example, the chimera may include one, two, three, or more substitutions in the DEN prM or E proteins. The chimera may also include one, two, three, or more substitutions in the WNV C protein, non-structural proteins, or non-coding regions. In particular examples, the chimeric flavivirus encodes a DEN2 E protein including N203D and an NS2A protein including I49T and F94L. In other examples, the
15 chimeric flavivirus encodes a DEN2 E protein including K122I, S186F, and N203D, a WNV NS2A protein including I49T and F94L, and a WNV NS4B protein including T241I.

The disclosure also provides WN/DEN chimeras encoding at least one amino acid substitution in the E protein, wherein antibody cross-reactivity of the E protein
20 is measurably reduced. In some examples, the chimera encodes at least one amino acid substitution, for example at DEN2 E protein amino acid position 101, 106, 107, 108, 135, or a combination of two or more thereof. It is to be understood that substitutions at the equivalent E protein amino acid positions in DEN1, DEN3, or DEN4 are also contemplated. Furthermore, substitutions at the equivalent position
25 are contemplated even in situations where the wild type amino acid is different from the amino acid in the wild type DEN2. Particular amino acid substitutions include, but are not limited to, W101F, G106A, G106L, L107F, F108W, F108M, F108V, F108L, L135W, or L135K. Additional examples of amino acid substitutions which reduce antibody cross-reactivity of flavivirus E proteins are known in the art (see
30 *e.g.* WO06/025990; incorporated herein by reference). Chimeras that include mutations that reduce E protein antibody cross-reactivity may also include one or

more additional mutations in the structural proteins, non-structural proteins, or NCRs, such as those described above.

Methods to assess the characteristics of the above-described WN/DEN chimeric viruses including sequence variants are well-known in the art. For example, methods of assessing viral titer, replication rate, plaque size, and stability in culture may be assessed as described in Example 2. See also, Obijeski *et al.*, *J. Gen. Virol.* 22:21-33, 1974; Beaty *et al.*, *Diagnostic Procedures for Viral, Ricksettial, and Chlamydial Infections*, pp. 189-212, Lennette *et al.* (eds.), 7th Edition, American Public Health Association, 1995; *Virology Methods Manual*, Mahy and Kangro (eds.), Academic Press, 1996; Huang *et al.*, *J. Virol.* 77:11436-11447, 2003; Huang *et al.*, *J. Virol.* 79:7300-7310, 2005. Methods to assess infectivity in mammals (such as mice) or mosquitoes can be carried out as described below (such as Examples 4, 6, and 7).

Reduction in antibody cross-reactivity can be determined by comparing antibody binding affinity of an antibody for the wild type E protein with antibody binding affinity for an E protein including one or more amino acid substitutions. A reduction in antibody binding affinity indicates a reduction in antibody cross-reactivity. Antibody binding affinities can be determined by many methods well known in the art, such as end-point titration in an Ag-ELISA assay, competition binding in an ELISA assay, a solid-phase radioimmunoassay, and the Biacore® surface plasmon resonance technique (Malmqvist, *Biochem. Soc. Trans.* 27:335-40, 1999; and Drake *et al.*, *Anal. Biochem.* 328:35-43, 2004). In some embodiments the antibody is a polyclonal antibody or a mAb. A specific, non-limiting example of a polyclonal antibody is polyclonal anti-DEN2 murine hyperimmune ascitic fluid. Specific, non-limiting examples of mAbs include 4G2 (ATCC No. HB-112), 6B6C-1, 1B7-5, 1A1D-2, 1A5D-1, 1B4C-2, F4540, D1-11, 9F10, D2811, 2H3, 9A3D-8, 3H5, 1F1, 8A1, or 1H10 (see, *e.g.*, Roehrig *et al.*, *Virology* 246:317-28, 1998; Crill and Chang, *J. Virol.* 78:13975-13986, 2004). Antibody cross-reactivity may be assessed as described in Example 10.

30

V. Preparation of Viruses and Virus Particles

Methods of cell culture, viral replication, plaque titration, and virus or virus particle purification are well known in the art. See *e.g.* Obijeski *et al.*, *J. Gen. Virol.* 22:21-33, 1974; Beaty *et al.*, *Diagnostic Procedures for Viral, Ricksettial, and*
5 *Chlamydial Infections*, pp. 189-212, Lennette *et al.* (eds.), 7th Edition, American Public Health Association, 1995; *Virology Methods Manual*, Mahy and Kangro (eds.), Academic Press, 1996.

The chimeric viruses of the present invention can be made using standard methods known and recognized in the art. For example, an RNA molecule
10 corresponding to the genome of a virus, or a chimeric virus, can be introduced into primary cells, chick embryos, or diploid cell lines, from which (or the supernatants of which) progeny virus can then be purified. Another method that can be used to produce the viruses employs heteroploid cells, such as Vero cells (Yasumura *et al.*, *Nihon Rinsho* 21:1201-1215, 1963). In this method, a nucleic acid molecule (*e.g.*, an
15 RNA molecule) corresponding to the genome of a virus or chimeric virus is introduced into the heteroploid cells, virus is harvested from the medium in which the cells have been cultured, and harvested virus is treated with a nuclease (*e.g.*, an endonuclease that degrades both DNA and RNA, such as Benzonase; U.S. Pat. No. 5,173,418). The nuclease-treated virus is then concentrated (*e.g.*, by use of
20 ultrafiltration using a filter having a molecular weight cut-off of, *e.g.*, 500 kDa (*e.g.*, a Pellicon-2 Mini ultrafilter cassette)), diafiltered against MEME without phenol red or FBS, formulated by the addition of lactose, and filtered into a sterile container. Details of a method of virus production are provided in WO 03/060088. Virus particles may be purified as discussed herein (see, *e.g.*, section VIII), for example,
25 by ultracentrifugation through a sucrose gradient and sucrose cushion.

VI. Detection of Flavivirus Antibodies

The present disclosure further provides a method of detecting a flavivirus-reactive antibody in a sample (such as a sample from a subject, for example, a blood
30 sample), including contacting the sample with a chimeric virus of this disclosure under conditions whereby an antibody/polypeptide complex can form; and detecting formation of the complex, thereby detecting flavivirus antibody in a sample. An

advantage of the disclosed WN/DEN chimeras is that they grow faster and to higher titers and produce larger plaques than wild type DENV. Therefore, the disclosure provides methods of detecting DENV-reactive antibody in a sample that are faster and more specific than methods utilizing wild type DENV. For example, the
5 specificity of the assay (for example to distinguish between DENV serotypes) may be improved by use of the disclosed chimeras which include amino acid substitutions in the E protein which reduce antibody cross-reactivity.

The method of detecting flavivirus-reactive antibody in a sample can be performed, for example, by contacting a fluid or tissue sample from a subject with a
10 chimeric virus of this disclosure and detecting the binding of at least one polypeptide encoded by the virus to the antibody. A fluid sample of this method can include any biological fluid which could contain the antibody, such as cerebrospinal fluid, blood, bile plasma, serum, saliva and urine. Other possible examples of body fluids include sputum, mucus and the like.

15 In one example, the presence of a Dengue virus antibody can be detected in a sample from a subject utilizing a disclosed chimeric flavivirus in a plaque-reduction neutralization test (PRNT) assay (see *e.g.*, Example 9). In the PRNT assay, a sample is contacted with a virus encoded by a chimeric flavivirus disclosed herein (such as a WN/DEN2 virus). A suitable cell culture (such as Vero, C6/36, or
20 BHK cells) is inoculated with the virus-sample mixture to infect the cells. The cell culture is incubated under conditions sufficient to allow plaque formation and the number of plaques formed in a culture inoculated with the chimeric virus-sample mixture is compared to the number of plaques formed in a control culture (such as cells inoculated with virus alone). A reduction in the number of plaques in the cell
25 culture inoculated with the chimeric virus-sample mixture as compared to the control culture (for example a decrease of at least 50%, 60%, 70%, 80%, 90%, 95%, or 99% compared with the control sample) indicates the presence of a DENV neutralizing antibody in the sample.

Enzyme immunoassays such as IFA, ELISA and immunoblotting can be
30 readily adapted to accomplish the detection of flavivirus antibodies in a sample according to the methods of this disclosure. An ELISA method effective for the detection of the antibodies can, for example, be as follows: 1) bind the chimeric

virus or virus particles to a substrate; 2) contact the bound chimeric virus with a fluid or tissue sample containing the antibody; 3) contact the above with a secondary antibody bound to a detectable moiety which is reactive with the bound antibody (for example, horseradish peroxidase enzyme or alkaline phosphatase enzyme); 4) contact the above with the substrate for the enzyme; 5) contact the above with a color reagent; and 6) observe/measure color change or development.

The detectable moiety allows for visual detection of a precipitate or a color change, visual detection by microscopy (such as a chromogenic deposit or fluorescence), or automated detection by spectrometry, radiometric measurement or the like. Examples of detectable moieties include fluorescein, fluorescein isothiocyanate, rhodamine, Cy5, and Cy3 (for fluorescence microscopy and/or the microsphere-based immunoassay), horseradish peroxidase (for either light or electron microscopy and biochemical detection), biotin-streptavidin (for light or electron microscopy) and alkaline phosphatase (for biochemical detection by color change).

Another immunologic technique that can be useful in the detection of flavivirus antibodies uses mAbs for detection of antibodies specifically reactive with flavivirus polypeptides in a competitive inhibition assay. Briefly, a sample is contacted with a chimeric flavivirus or virus particle of this invention which is bound to a substrate (for example, a 96-well plate). Excess sample is thoroughly washed away. A labeled (for example, enzyme-linked, fluorescent, radioactive, *etc.*) mAb is then contacted with any previously formed polypeptide-antibody complexes and the amount of mAb binding is measured. The amount of inhibition of mAb binding is measured relative to a control (no antibody), allowing for detection and measurement of antibody in the sample. The degree of mAb binding inhibition can be a very specific assay for detecting a particular flavivirus variety or strain, when based on mAb binding specificity for a particular variety or strain of flavivirus. mAbs can also be used for direct detection of flavivirus in cells by, for example, IFA according to standard methods.

As a further example, a micro-agglutination test can be used to detect the presence of flavivirus antibodies in a sample. Briefly, latex beads, red blood cells or other agglutinable particles are coated with a chimeric flavivirus or virus particles of

this disclosure and mixed with a sample, such that antibodies in the sample that are specifically reactive with the antigen crosslink with the antigen, causing agglutination. The agglutinated antigen-antibody complexes form a precipitate, visible with the naked eye or measurable by spectrophotometer.

5 In yet another example, a microsphere-based immunoassay can be used to detect the presence of flavivirus antibodies in a sample. Briefly, microsphere beads are coated with a chimeric flavivirus or virus particle of this disclosure and mixed with a sample, such that antibodies in the sample that are specifically reactive with an antigen encoded by the virus bind the antigen. The bead-bound virus-antibody
10 complexes are allowed to react with fluorescent-dye labeled anti-species antibody (such as FITC-labeled goat anti-human IgM), and are measured using a microsphere reader (such as a Luminex instrument).

VII. Evaluation of Candidate Vaccine Efficacy

15 The chimeric flaviviruses disclosed herein may be used in methods to assess the efficacy of candidate vaccines, such as DENV vaccine candidates. A number of candidate DENV vaccines have been developed previously, such as attenuated vaccine strains (for example DEN2 PDK-53, DEN1 PDK-13, DEN3 PGMK-30/FRhL-3, and DEN4 PDK-48) and chimeric DENV constructs (see *e.g.* U.S. Pat.
20 No. 7,094,411). However, currently there is no ideal mouse model for evaluation of candidate DENV vaccines, because outbred immune competent mice do not succumb to wild type DENV challenge and do not generate sufficient viremia for measuring a protective effect of a candidate vaccine.

 The efficacy of candidate DENV vaccines may be tested by inoculating
25 subjects (for example, mice or non-human primates (such as rhesus monkeys)) with a candidate vaccine, followed by challenge with a virulent DENV strain. The disclosed WN/DENV chimeras may be virulent and/or generate significant viremia in non-immunized mice, therefore they can be used as the challenge dose in previously inoculated subjects.

30 In one particular embodiment, a set of subjects (such as mice) is inoculated with a candidate DENV vaccine (for example, DENV2 PDK-53 strain). Administration of the candidate vaccine strain virus may be carried out by any

suitable means, including both parenteral injection (such as intraperitoneal, subcutaneous, or intramuscular injection), by *in ovo* injection in birds, orally, and by topical application of the virus (typically carried in the pharmaceutical formulation) to an airway surface. Topical application of the virus to an airway surface can be carried out by intranasal administration (e.g. by use of dropper, swab, or inhaler which deposits a pharmaceutical formulation intranasally) or by inhalation administration, such as by creating respirable particles of a pharmaceutical formulation (including both solid particles and liquid particles) containing the virus as an aerosol suspension, and then causing the subject to inhale the respirable particles. In a particular example, the subjects are inoculated intraperitoneally with vaccine virus in a vehicle such as phosphate buffered saline. Multiple inoculations (such as boosters) may be carried out, separated by a suitable period of time, such as at least two weeks, four weeks, eight weeks, twelve weeks, or more.

Subjects that have been test vaccinated are challenged with a virulent or lethal dose (such as a lethal dose determined as in Example 8) of a flavivirus chimera disclosed herein (for example a WN/DEN chimera, such as that encoded by one of SEQ ID NOs: 1, 3, 5, or 7) following a suitable period of time to allow immunity based on the vaccination to develop (such as at least two weeks, four weeks, eight weeks, twelve weeks, or more). The challenge dose may be administered by any suitable route including those above, and optionally is administered by the same or a different route as the vaccinating dose. Following the challenge dose, subjects are monitored for development of morbidity (such as fever, rash, vomiting, loss of appetite, rough fur, hunched back, lethargy, unbalanced or irritable movement, dehydration, weight loss, or signs of paralysis) or mortality. In addition, blood is collected from subjects after challenge for measurement of viremia levels. A decrease in viremia levels, signs of morbidity and/or mortality compared to a set of control subjects which is not inoculated with the candidate vaccine (for example, a decrease of at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, or 99% in a test vaccinated population compared with a control population) indicates the effectiveness of the candidate vaccine.

VIII. Production of Purified Virus Particles Containing Dengue prM and E Proteins

The chimeras disclosed herein may also be used to rapidly produce quantities of virus particles containing DENV prM (or M) and E proteins, as wild type and
5 attenuated DENV strains generally do not replicate efficiently in culture.

Methods of protein purification are well-known in the art (see *e.g.* Scopes, *Protein Purification: Principles and Practice*, 3rd edition, Springer, 1994).

Examples of methods of protein purification include immunoaffinity purification, ultracentrifugation over a gradient, ion exchange chromatography, and gel filtration.

10 In one example, a method is disclosed of producing virus particles containing Dengue virus E and prM (or M) proteins. The method includes producing the chimeric flaviviruses disclosed herein in a cell culture system, such as Vero, C6/36, LLC-MK₂, or BHK cells. The cells are infected with a WN/DEN chimera and incubated for sufficient time for virus to be produced (for example, at least 2 days, 3
15 days, 4 days, 5 days, 8 days, or 10 days). A supernatant, such as the cell culture medium, containing the chimeric virus is collected and condensed by PEG-precipitation. The virus particles are then purified through ultracentrifugation over a gradient (such as sucrose or glycerol/potassium tartrate gradient). See *e.g.*, Obijeski *et al.*, *J. Gen. Virol.* 22:21-33, 1974. In a particular example, Vero or C6/36 cells are
20 infected with the disclosed WN/DEN2 chimera and the viral particle with DEN2 prM and/or E protein is purified.

Purified virus particles containing DENV prM and E proteins are suitable for use in place of proteins prepared by other means (such as recombinant expression in mammalian cells, yeast, or *E. coli*). For example, purified virus particles of the
25 disclosed chimeric viruses with DENV prM and/or E proteins may be used in methods of detecting antibodies against these proteins (such as diagnostic tests or assays to determine response to a candidate vaccine). For example, purified virus particles may be immobilized on a solid support and utilized in immunodetection methods such as ELISA, competitive inhibition assays, micro-agglutination test, or
30 microsphere based immunoassays. Further, the purified virus particles are suitable for use in PRNT assays for detection of neutralizing DENV antibodies.

Further, because the disclosed WN/DEN chimeric viruses grow more rapidly in culture and to higher titers than DEN viruses, purified virus particles containing DENV prM and/or E proteins are useful for production of DENV antigens. Uses for these antigens include production and testing of vaccine candidates and use of virus
5 particles for further study of protein folding, three-dimensional structure, and epitope mapping.

IX. Mosquito Infectivity and Transmissibility

Aedes aegypti mosquito is the major vector for DENV, while *Culex*
10 *quinquefasciatus* and *Culex pipiens* mosquitoes are the natural vectors for WNV. The disclosed chimeric flaviviruses may have reduced or eliminated infectivity and/or transmissibility in one or more mosquito vectors. Methods for determining whether a virus can infect or be transmitted by a mosquito species or strain are known to one of skill in the art.

15 By way of example, to determine if the disclosed chimeric viruses can infect *A. aegypti*, *C. quinquefasciatus*, *C. pipiens*, or other mosquito species, mosquitoes can be fed bloodmeal containing virus (such as a 1:1 mixture of cell supernatant from infected cells and defibrinated calf or sheep blood) or by intrathoracic inoculation with medium containing virus (such as about 10^5 to 10^7 pfu).

20 Infected or control mosquitoes are cold anesthetized and dissected. Midguts and/or heads are collected and fixed in either acetone (heads) or 4% paraformaldehyde (midguts) and stained by immunofluorescence assay with a pan-flavivirus E protein antibody, such as 4G2, or serotype-specific antibodies, such as 3H5 (DEN2), 1F1 (DEN1), 8A3 (DEN3), or 1H10 (DEN4). A fluorescein-
25 conjugated antibody (such as goat anti-mouse IgG antibody) is used for secondary detection. Tissue immunofluorescence assays are read using a fluorescent microscope.

Decreased E protein immunofluorescence in mosquitoes infected with chimeric WN/DEN virus as compared to control samples (those infected with
30 DENV or WNV) indicates that the chimeric virus has decreased infectivity and/or transmissibility in a particular mosquito species as compared to the wild type DENV or WNV. An increase in E protein immunofluorescence in mosquitoes infected with

chimeric WN/DEN virus as compared to control samples (such as those infected with DENV or WNV) may indicate that the chimeric virus has increased infectivity and/or transmissibility in a particular mosquito species as compared to the wild type DENV or WNV.

5

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

10

EXAMPLES

Example 1

Construction of WN/DEN2 Chimeric Virus

This example describes construction of a chimeric West Nile/Dengue-2 virus consisting of the prM and E genes from DEN2 in a WNV backbone.

15

A WNV NY99 infectious clone (designated as WN/IC-P991 clone) in a two plasmid system was used. pWN-AB-Asc contained nucleotides 1 to 2495 and pWN-CG contained nucleotides 2495-11029 (described in Kinney *et al.*, *J. Gen. Virol.* 87:3611-3622, 2006). Site-directed mutagenesis of pWN-AB-Asc was performed using the QuikChange® Multi site-directed mutagenesis kit (Stratagene, La Jolla, CA) to create a SacII site at nucleotides 412-418 and a BspEI site at nucleotides 2424-2429 of the WNV genome. Using similar site-directed mutagenesis, these sites were engineered in a DEN2 16681 infectious clone (D2/IC-30P-A; Kinney *et al.*, *Virology* 230:300-308, 1997) to generate D2IC-30P-NBX clone. Restriction digestion with SacII and BspEI was used to cut the prM and E genes (bp 398-2430) out of both the DEN2 16681 and WNV infectious clones. The prM (including the DEN2 prM signal sequence at the end of the C gene, which serves as an anchor for the C protein during polyprotein processing) and E genes of DEN2 16681 was ligated in the pWN-AB-Asc plasmid, replacing the WNV prM and E genes with the DENV equivalents, to create pWN/D2-AB.

25
30

Full length genomic cDNA was prepared by cleaving pWN/D2-AB and pWN-CG at the natural NgoMIV site located at bp 2495 of the WNV genome. The two plasmids were then ligated together at the NgoMIV site and transcribed using

the AmpliScribe™ T7 kit (Epicentre Technologies, Madison, WI). Figure 1 shows a schematic diagram of the WN/DEN2 chimera (WN/D2) and the junction between the WN C protein and the DEN2 prM signal sequence and prM protein.

In vitro transcription was carried out at 37° C for 2-3 hours and C6/36 or
5 Vero cells were transfected with the transcribed RNA by electroporation. Infectious virus was collected 4-7 days post transfection. Virus was designated as C6/36-0 seed when recovered from transfected C6/36 cells, and as Vero-0 when recovered from transfected Vero cells. The C6/36-0 and Vero-0 seeds were used to infect C6/36 and Vero cells, respectively, to obtain the C6-1 and V-1 working seeds.

10 To verify the genome accuracy of the recovered chimeras, viral RNA was extracted from C6-1 virus using the QIAamp® Kit (Qiagen Inc, Valencia, CA) and cDNA products were generated using the Titan™ One-Tube RT-PCR system (Roche Applied Science, Indianapolis, IN). PCR products were directly sequenced to confirm the genome sequence of the chimeric WN/DEN2 virus (SEQ ID NO: 1).

15

Example 2

WN/DEN2 Chimeric Virus Replication and Titration in Cells

This example describes the replication of a WN/DEN2 chimeric virus in C6/36 and Vero cell lines.

20 *Methods*

Three T75 flasks of C6/36 and Vero cells were infected at a multiplicity of infection (MOI) of 0.001 with each of the following viruses: WN NY99, DEN2 16681, or WN/DEN2 virus (produced as described in Example 1). Viruses in either 3 ml of Ye-Lah medium (10 g yeast extract and 50 g lactalbumin hydrolysate per
25 1000 ml water) containing 2% fetal calf serum (C6/36 cells) or 3 ml of Iscove's Modified DMEM (*e.g.* Gibco-Invitrogen, Carlsbad, CA) containing 2% fetal calf serum (Vero cells) were added to the monolayer when the cells were 95-100% confluent. Following infection, flasks were incubated at 28°C and 37°C for C6/36 and Vero cells, respectively. Flasks were rocked every 15 minutes for 1.5 hours for
30 viral adsorption. After 1.5 hours, 27 ml of growth medium was added to each flask and the flasks were incubated at 28°C for C6/36 cells or 37°C for Vero cells for 10 days. Every 2 days an aliquot of 300 µL was removed from each flask and

combined with 300 μ L of Iscove's DMEM containing 35% fetal calf serum. All aliquots were stored at -80°C until they could be titrated by plaque assay on Vero cells. After plaque titration of each aliquot, the viral growth kinetics in each cell type was established, and also used to determine the proper seed harvest day when
5 producing chimera seeds. When producing chimeric virus seeds, the culture conditions were proportionally scaled up to larger tissue culture vessels.

Virus samples were titrated by plaque assay on Vero cells. Serial 10-fold dilutions of all virus samples were made in BA-1 diluent. 100 μ L of each dilution was added to one well of a 6-well tissue culture plate containing a confluent
10 monolayer of Vero cells. Following inoculation, plates were incubated at 37°C in an atmosphere of 5% CO₂ for 1.5 hours to allow virus adsorption. Plates were rocked every 10 minutes to ensure that the monolayer did not dry out. After virus adsorption all wells were overlaid with 4 ml of 0.8% agarose overlay containing a balanced salt solution and Ye-Lah medium. Plates were incubated at 37°C in an
15 atmosphere of 5% CO₂ for the indicated times. Following incubation, wells were overlaid with another 2 ml of agarose overlay that also contains 1.5% neutral red to visualize plaques. Plates were read and plaques were counted the following day.

Plaque isolation and serial passage of the chimera in Vero cells was performed. One of the large plaques from the V-1 seed (as V-2) was isolated from
20 the titration plate, and used to infect a flask of fresh Vero cells. The culture medium harvested from this flask was designated as V-3 seed, and was serially passaged through Vero cells seven more times to obtain the V-4 to V-10 seeds. Additionally, the plaques produced from the C6-1 working seed in the Vero plaque titration plate also showed a small portion of larger plaques among the pin-point size plaques.
25 One large plaque was isolated from the plate (as C6-1/V-1) and used to infect a flask of fresh Vero cells to obtain C6-1/V-2 seed.

PRNT tests were performed by incubating viruses with serial dilutions of a WNV hyperimmune mouse ascitic fluid (HMAF; M28548), a WNV E protein-specific monoclonal antibody (MAb3.67G), a DEN2 HMAF (VS0090), or a DENV-
30 2 E protein-specific monoclonal antibody (MAb3H5) at 4 °C overnight. The following day, samples were added to Vero cells in 6-well culture plates and followed by overlay procedure as in the plaque titration method described above.

Neutralization titer was determined as the greatest antibody dilution that decreased plaques by at least 50% compared to the back titration results of the input virus in the same assay.

Results

5 The WN/DEN2 chimeric virus replicated efficiently and reached high titers in C6/36 cells (8.6 log₁₀ pfu/ml for C6-1 seed). Plaque formation was also assessed in Vero cells. Titers of the working seed after one passage in Vero cells (V-1 seed) was about 4-5 log₁₀ pfu/ml. Compared to the plaque size of the parental D2 16681 and WN NY 99 viruses in Vero cells, the chimeric WN/DEN2 virus formed plaques
10 that were much larger than the D2 16681 virus on day 8 post-infection (p.i.) (Figure 2), but still smaller than the fast growing WN NY99 virus, which would lyse the whole cell sheet on day 8 post infection.

 Plaques were visualized and measured on day 5 p.i. after passage in C6 or Vero cells (Figure 3). Wild type West Nile NY99 virus (WNV NY99) and DEN-2
15 16681 virus were included in the same experiment for comparison. WNV NY99 produced large plaques at day 5 p.i., while no plaques were visible for DEN-2 16681 at this time point. Plaque size increased following additional passages in Vero cells (Table 3).

 Full genome sequence was obtained for the C6-1 working seed as well as the
20 serial Vero passage seeds, V-3, V-10, and C6-1/V2. The consensus sequence of the C6-1 seed showed no mutation compared to the parent West Nile and DEN-2 viruses from which it was derived. The C6-1/V-2 seed contained two silent mutations (nucleotide 3291 of SEQ ID NO: 1 C>T and 8469 of SEQ ID NO: 1 A>G) and one missense mutation (nucleotide 1558 of SEQ ID NO: 1; A>G),
25 resulting in an amino acid change from N to D at position 203 of the DEN2 E protein (amino acid 488 of SEQ ID NO: 2; E-N203D). The V-3 passage had two silent mutations (nucleotide 1566 of SEQ ID NO: 1 T>C and nucleotide 2973 of SEQ ID NO: 1 T>C) and three missense mutations, nucleotide 1558 of SEQ ID NO: 1 (A>G), nucleotide 3638 of SEQ ID NO: 1 (T>C), and nucleotide 3772 of SEQ ID
30 NO: 1 (T>C). These missense mutations resulted in amino acid substitutions in the DEN2 E protein E-N203D (amino acid 488 of SEQ ID NO: 2) and the WNV NS2A protein, NS2A-I49T (amino acid 1181 of SEQ ID NO: 2) and NS2A-F94L (amino

acid 1226 of SEQ ID NO: 2). The V-10 seed showed 4 silent mutations (nucleotide 1566 of SEQ ID NO: 1 T>C, nucleotide 2973 of SEQ ID NO: 1 T>C, nucleotide 3600 of SEQ ID NO: 1 T>T/C mix, and nucleotide 6181 of SEQ ID NO: 1 C>C/T mix) and 6 missense mutations (nucleotides 1316 A>T/A, 1508 C>T, 1558 A>G
 5 3638 T>C, 3772 T>C, and 7604 C>T, all numbered as in SEQ ID NO: 1). The resulting amino acid changes are shown in Table 3. Two of the missense mutation loci had mixed nucleotides, resulting in a mixed genotype of E-K122I/K (amino acid 407 of SEQ ID NO: 2) and NS4B-T241T/I (amino acid 2503 of SEQ ID NO: 2).

The E-N203D mutation was found in two seeds (C6-1/V-2 and V-3) that
 10 were descendents from separate virus seeds derived from independent experiments. This indicates that this particular mutation may be critical for the chimera adapting to the Vero cell cultures. This mutation may also slightly increase the plaque size of the chimera in Vero cells, further supporting its effect on virus growth in Vero. In addition, the two NS2A mutations in the V-3 seed, NS2A-I49T and NS2A-F49L,
 15 also significantly increased the virus growth in Vero cells, resulting in larger plaques compared to the C6-1/V-2 seed. The E-K122I mutation (which eliminates the positive charge at E protein amino acid 122) may also be a Vero cell-adapting mutation for DEN2.

20 **Table 3. Plaque size and sequence of successive passages of WN/DEN2**

Virus	Passage History	Amino Acid Mutations		Plaque Size at Day 5 p.i. (mm)*
		Protein	Mutation	
WNV NY99	LLC-MK2-1	N/A	N/A	5.18 +/- 0.37
DENV-2 16681	C6-1	N/A	N/A	Could not be visualized
WN/D2	C6-1	None	None	0.48 +/- 0.08
WN/D2	C6-1/V-2	E	N203D	1.05 +/- 0.16
WN/D2	V-3	E NS2A NS2A	N203D I49T F94L	1.55 +/- 0.20
WN/D2	V-10	E E E NS2A NS2A NS4B	K122I/K S186F N203D I49T F94L T241T/I	1.78 +/- 0.43

* mean $\pm$ standard deviation

N/A: not applicable

The growth kinetics of the wild type and chimeric viruses were tested in both Vero and C6/36 cells. Vero cells were infected with chimeric WN/DEN2, WNV NY99, or DENV-2 16681 at the same multiplicity of infection (MOI) and titer was
5 determined for 10 days (Figure 4). Wild type WNV NY99 reached its maximum titer ($8.8 \log_{10}$ pfu/ml) by day 2 and the titer dropped rapidly in subsequent days. WN/DEN2 V-10 seed also reached its maximum titer by day 2 ($6.3 \log_{10}$ pfu/ml), but only decreased slightly in titer by day 10. WN/DEN2 V-3 and C6-1/V-2 seeds approached their maximum titers ($5.5 \log_{10}$ pfu/ml and $5.6 \log_{10}$ pfu/ml,
10 respectively) by day 4 and then remained fairly consistent (with a slight overall increase) through day 10. WN/DEN2 C6-1 grew more slowly, reaching its maximum titer at day 6 ($84.8 \log_{10}$ pfu/ml). DENV-2 16681 also reached its maximum titer ($5.5 \log_{10}$ pfu/ml) at day 6 and remained fairly consistent throughout the remaining days.

15 Virus replication in C6/36 cells showed that chimeric WN/DEN2 viruses all reached peak titers of approximately $8.5 \log_{10}$ pfu/ml (Figure 5), similar to that of DENV-2 16681. WNV NY99 reached a peak titer of $9.6 \log_{10}$ pfu/ml. Except for the V-10 seed, all the chimeric viruses reached peak titers at day 6 p.i., similar to WNV NY99. The V-10 seed reached peak titer on day 8, and had a very similar
20 growth profile to that of DENV-2 16681.

Plaque reduction neutralization (PRNT) tests were performed on WNV NY99, DENV-2 16681, WN/DEN2 C6-1, WN/DEN2 C6-1/V-2, WN/DEN2 V-3, and WN/DEN2 V-10 to determine their antigenic profile (Table 4). Although, the endpoint of some of the PRNT titers was not determined, all the chimeras had a
25 similar neutralization pattern to the wt DENV-2 16681 by all 4 tested antibodies. The traditional PRNT of the DENV-2 16681 virus usually takes about 8-10 days due to the slow growth and tiny plaques produced by the virus. On the other hand, all the WN/D2 seeds tested showed plaques by day 5 p.i., resulting in faster PRNT. The similar PRNT patterns to those of wt DENV-2 and the rapid plaque forming
30 ability make WN/D2 chimeras suitable as a surrogate DENV-2 virus in diagnosis by PRNT.

Table 4. PRNT assay of WN/DEN2 chimeric viruses

Virus	PRNT ₅₀ Titer*			
	WNV HMAF (M28548)	WNV E-Specific MAb (MAb3.67G)	DENV-2 HMAF (VS0090)	DENV-2 E-Specific MAb (MAb3H5)
WNV NY99	10240	>40960	<160	<80
DENV-2 16681	<640	<1280	5120	>2560
WN/D2 C6-1	<640	<1280	>5120	>2560
WN/D2 C6-1/V-2	<640	<1280	5120	>2560
WN/D2 V-3	<640	<1280	5120	>2560
WN/D2 V-10	<640	<1280	>5120	>2560

* Greatest antibody dilution that decreased plaques by at least 50%.

Example 3

5 Characteristics of WN/DEN2 Chimeras with Specific Mutations

This example describes the *in vitro* characteristics of WN/DEN2 chimeras containing specific introduced mutations.

Methods

To further modify the chimeric WN/DEN2 virus for adapting to mammalian
 10 cell cultures, chimeric constructs WN/D2-E203 (containing E-N203D; amino acid
 488 of SEQ ID NO: 2), WN/D2-2A (containing NS2A-I49T and NS2A-F94L;
 amino acids 1181 and 1226 of SEQ ID NO: 2, respectively), and WN/D2-E-2A
 (containing E-N203D, NS2A-I49T and NS2A-F94L) were made. cDNA fragments
 containing the E-N203D mutation, or NS2A-I49T and NS2A-F94L mutations were
 15 RT-PCR amplified from the C6-1/V-2 seed or V-3 seed (described in Example 2),
 respectively. The fragment with E-N203D was cloned into the 5'-plasmid
 containing chimeric WN/DEN2 cDNA from nt 1-2445 (pWN/D2-AB) to obtain new
 mutant plasmid, pWN/D2-AB-E203. The NS2A mutant fragment (including the
 silent mutation at nucleotide 2973 of SEQ ID NO: 1 T>C) was cloned into the wt 3'-
 20 WN plasmid containing WNV cDNA nt 2440-10996 (pWN-CG) to obtain mutant
 pWN-CG-2A. WN/D2-E203 was constructed by ligating pWN/D2-AB-E203 with
 wt pWN-CG by NgoMIV junction (nt 2495 of WNV). WN/D2-2A was constructed
 by ligating wt pWN/D2-AB with pWN-CG-2A by NgoMIV junction. WN/D2-E2A

was made by ligating pWN-AB-E203 with wt pWN-CG-2A by NgoMIV junction. Each chimeric virus was recovered from transfected Vero cells and the working seed (V-1) of each virus was made after one passage of the transfection seed to Vero cells.

5 *Results*

Full genome sequencing of the V-1 seed confirmed that no additional missense mutations accrued during replication in Vero cells. Only a silent mutation at nt 2973 of SEQ ID No 1: (T>C) was found in both WN/D2-2A and WN/D2-E2A virus; this mutation was introduced during the cloning of pWN-CG-2A plasmid used
10 in the process of deriving these chimeras.

Plaque assays using each chimera showed that all produced larger plaques in Vero cells than the original WN/D2- C6-1 seed (Figure 6). Plaques produced by these chimeras could be easily visualized by day 4 p.i. There was a mixed plaque phenotype in the WN/D2-2A seed, which may indicate this virus was still evolving
15 in Vero cells. However, both chimeras with the E-N203D mutation appeared to be quite stable in Vero cells.

Example 4

Determination of Mosquito Infectivity of WN/DEN2 Chimera

20 This example describes the determination of the ability of WN/DEN2 chimera to infect DENV and WNV mosquito vectors.

Methods

Infectious bloodmeals were made using freshly prepared viruses with an approximate titer of 10^7 pfu/ml in a 1:1 ratio with defibrinated sheep blood. *Aedes*
25 *aegypti*, *Culex pipiens*, or *Culex quinquefasciatus* mosquitoes were allowed to feed on infectious bloodmeals provided in a HEMOTEK membrane feeder. Bloodfed mosquitoes were held at 28 °C for 10 days. Midguts and heads were dissected, fixed on slides, and stained via immunofluorescence using the pan-flaviviral monoclonal antibody 4G2.

30 *Results*

Aedes aegypti mosquito is the major vector for DENV, while *Culex quinquefasciatus* and *Culex pipiens* are the natural vectors for WNV. The

WN/DEN2 C6-1 chimera (which lacks any Vero cell-adapting mutations) infects the midgut of *Culex quinquefasciatus* and *Aedes aegypti* in a pattern more similar to that of wild type WNV than wild type DEN2 virus (Figure 7), suggesting the WNV non-structural genes in the chimera virus have significant effects in infection of these mosquitoes. However, the WN/DEN2 C6-1 virus had similar low infection rate as the DENV-2 in the midgut of *Culex pipiens*, suggesting the structural DENV2 genes in the chimera were controlling the infection in *Culex pipiens*.

Example 5

Construction of Additional WN/DEN Chimeric Viruses

This example describes the construction of chimeric viruses containing the WNV backbone and prM and E protein from DEN1, DEN3, or DEN4.

The prM (including the DEN4 prM signal sequence) and E genes of DEN4, was ligated in the pWN-AB-Asc plasmid, replacing the WNV prM and E genes with the DENV equivalents, to create pWN/D4-AB. Two chimeras were constructed, one with wild type WNV components and one including the WNV NS2A mutations I49T and F94L. Full length genomic cDNA was prepared by cleaving pWN/D4-AB and pWN-CG (or WN-CG-2A) at the natural NgoMIV site located at bp 2495 of the WNV genome. The two plasmids were then ligated together at the NgoMIV site and transcribed using the AmpliScribe™ T7 kit (Epicentre Technologies, Madison, WI). Figure 8 shows a schematic diagram of the WN/DEN4 chimera (WN/D4) and the junction between the WN C protein and the DEN4 prM signal sequence and prM protein. The nucleic acid and amino acid sequences of a WN/DEN4 chimera are provided in SEQ ID NOs: 7 and 8, respectively. The WN/DEN4 chimera having the NS2A mutations had amino acid substitutions at amino acids 1181 and 1226 of SEQ ID NO: 8, respectively.

WN/DEN1 and WN/DEN3 chimeras are created as above and the junction between the WN C protein and the DEN prM signal sequence and prM protein is shown in Figure 8. The nucleic acid and amino acid sequences of a WN/DEN1 chimera are provided in SEQ ID NOs: 3 and 4, respectively. The WN/DEN1 chimera having the NS2A mutations had amino acid substitutions at amino acids 1181 and 1226 of SEQ ID NO: 4, respectively. The nucleic acid and amino acid

sequences of a WN/DEN3 chimera are provided in SEQ ID NOs: 5 and 6, respectively. The WN/DEN3 chimera having the NS2A mutations had amino acid substitutions at amino acids 1179 and 1224 of SEQ ID NO: 6, respectively.

5

Example 6

Assessment of Neurovirulence and Neuroinvasion Kinetics of Chimeric Virus

This example describes methods for assessing the neurovirulence and neuroinvasion kinetics of WN/DEN chimeric viruses (such as produced in Examples 1 or 5) in mice.

10 *Neurovirulence*

Groups of 10 mice (such as Swiss Webster, NIH Swiss, or ICR mice) are inoculated intracranially with ten-fold dilutions of WNV and WN/DEN chimeric virus from 0.1 pfu to 1000 pfu. One group of 10 mice is inoculated with 1000 pfu of DENV. The virus is diluted in 30 μ L of sterile phosphate buffered saline (PBS) and is administered via intracranial inoculation. Mice are monitored daily for 4 weeks to determine the virulent dose. Mice showing signs of illness (such as rough fur, hunched back, lethargy, unbalanced or irritable movement, dehydration, 10% weight loss, or signs of paralysis) are euthanized. The results are used to calculate the 50% virulent dose (VD₅₀) of the WNV/DENV chimera. A decreased VD₅₀ compared to DEN wild type virus indicates higher neurovirulence than the wild type DENV.

Viremia/Neuroinvasion

Groups of 12 mice (such as Swiss Webster, NIH Swiss, or ICR mice) are inoculated with 1000 pfu of WNV, DENV, and WN/DEN chimera. Virus is inoculated intraperitoneally in 100 μ L of PBS. At days 1, 3, 5, and 7 p.i., mice from each group are sacrificed and blood and brain samples are collected from each mouse. Brains are homogenized in DMEM and both blood and brain titers are determined by plaque assay on Vero cells, as described in Example 2. An increased number of pfu as compared to a control sample (such as a DENV or WNV) indicates increased neuroinvasion or virulence. A decreased number of pfu compared to a control sample (such as a DENV or WNV) indicates decreased neuroinvasion or virulence. Chimeras with higher neuroinvasion are further evaluated in 12-week-old

mice for developing a surrogate DENV dose for virulence challenge study as described in Example 8.

Example 7

5 **Determination of Antibody Response to WN/DEN Chimeras**

This example describes methods for the determination of antibody responses in mice or other animals inoculated with WN/DEN chimeras. Antibodies to DENV prM and E proteins and WNV non-structural proteins, particularly NS1, are measured. This example also describes a method for assessing the protective
10 efficacy of immune response to WNV non-structural proteins.

Prior to virus inoculation, pre-immune serum blood samples are collected from all mice by nicking the tail vein. Groups of 10 mice (such as Swiss Webster, NIH Swiss, or ICR mice) are inoculated intraperitoneally with 100 μ L of PBS containing serial ten-fold dilutions of WNV or WN/DEN chimeric virus ranging
15 from 0.1 to 1000 pfu. One group of 10 mice is inoculated with 1000 pfu of DENV. Mice are monitored daily for four weeks and mice showing signs of illness will be euthanized. Four weeks after primary virus inoculation, blood is collected from all surviving mice by nicking the tail vein. Only low dose groups of WNV and chimeric WN/DEN inoculated mice, and mice inoculated with 1000 pfu of DENV
20 are expected to survive. Serum samples from the collected blood are heat-inactivated at 56°C for 30 minutes and antibodies in the serum are determined by ELISA and/or PRNT assays.

Two days after blood collection, all mice are inoculated with a lethal dose of WNV NY99 (1000 pfu). The virus is delivered in 100 μ L of PBS via intraperitoneal
25 inoculation. Mice are monitored daily and moribund mice are euthanized by overexposure to CO₂ gas. Blood is collected from mice surviving more than 21 days after WNV challenge and antibodies in serum are determined by ELISA and/or PRNT. Antibody responses and survival ratios in the groups that are first inoculated with chimeric WN/DEN virus are used to evaluate the protective efficacy of the
30 immune response triggered by WNV non-structural proteins from the chimeric WN/DEN virus. Increased antibody response and/or survival ratio in animals inoculated with WN/DEN chimeric virus and challenged with WNV indicates that

an antibody response and protective immunity are the result of WNV C protein or non-structural proteins.

Example 8

5 **Determination of WN/DEN Lethal Dose and Evaluation of DENV Vaccine Efficacy**

This example describes methods for using WN/DEN chimeric viruses to evaluate the efficacy of candidate DENV vaccines.

The lethal dose of WN/DEN chimeric virus is determined in mice by
10 inoculating 12 week old mice (such as Swiss Webster, NIH Swiss, or ICR mice; 8 animals per group) with PBS as a control, or 10, 100, 1000, or 10,000 times the VD_{50} calculated from the neuroinvasion experiment (described in Example 6) or 10^4 , 10^5 , 10^6 , or 10^7 pfu, whichever is lower. Mice are inoculated intraperitoneally with virus in 100 μ L of PBS. Mice are monitored for signs of clinical illness daily and
15 moribund mice are euthanized by overexposure to CO_2 gas. The VD_{50} (the dose causing sickness in 50% of the mice inoculated) is calculated and the lethal dose is generally 10-1000 x VD_{50} . Usually 100 x VD_{50} is used. Alternatively, blood can be collected after virus challenge to determine the viremia levels from each group. Chimeric virus doses causing higher viremia compared to a wild type DENV control
20 group (such as 100-fold, 1000-fold, 10000-fold or higher) may be used for vaccine efficacy study.

To assess candidate DENV vaccine efficacy, groups of 4 week old mice (such as Swiss Webster, NIH Swiss, or ICR mice) are inoculated intraperitoneally with wild type DENV, DENV vaccine strain, or PBS. Mice in each group are
25 inoculated intraperitoneally with 10^5 pfu of virus in 100 μ L of PBS. Identical immunizations are given four to six weeks later. The mice are given WN/DEN chimeric virus at a lethal dose (100 x VD_{50}) or a dose causing high viremia compared to wild type DENV when they are 12 weeks old. Mice are bled to determine viremia level and monitored daily for signs of morbidity after lethal virus
30 challenge. Mice with the first sign of morbidity, such as rough fur, hunched back, lethargy, unbalanced or irritable movement, dehydration, 10% weight loss, or signs of paralysis are euthanized immediately by overexposure to CO_2 gas.

Blood is also collected prior to secondary immunization, lethal WN/DEN challenge, and from mice surviving 21-28 days after lethal challenge. Antibodies to DEN or WNV proteins in the serum are determined by PRNT. Protective efficacy of the vaccine is evaluated by comparing viremia levels or survival ratios of the vaccinated groups to the non-immunized control group. Increased survival, decreased viremia level, or increased anti-DENV antibody production of mice inoculated with a candidate vaccine as compared to a control group indicates a DENV vaccine candidate suitable for further testing. No increase in survival or decrease in viremia level compared to a non-immunized control group indicates poor protective efficacy by the DENV vaccine candidate.

Example 9

Neutralizing Antibody Assays

This example describes methods of assessing neutralizing antibody response to DENV infection or WN/DEN virus chimeras using a plaque-reduction neutralization assay (PRNT) or immunostaining-based neutralization assay.

Serum samples are tested for neutralizing antibodies by serum-dilution PRNT. 60-100 pfu of WN/DEN virus chimera or wild type DENV is incubated with serial 2-fold dilutions of heat-inactivated (56°C for 30 minutes) serum specimens overnight at 4°C. The virus-serum mixtures are inoculated in tissue culture plates containing a confluent monolayer of Vero cells. Following inoculation, plates are incubated at 37°C in an atmosphere of 5% CO₂ for 1.5 hours to allow virus adsorption. Plates are rocked every 10 minutes to ensure the cell monolayer does not dry out. After virus adsorption, the cells are overlaid with 0.8% agarose containing balanced salt solution and Ye-Lah medium (10 g yeast extract and 50 g lactalbumin hydrolysate per 1000 ml water). Plates are incubated at 37°C in an atmosphere of 5% CO₂ for 1-7 days. Following this incubation, wells are overlaid with another agarose overlay containing 1.5% neutral red to visualize plaques. Plaques are counted the following 1-3 days.

A reduction in the number of plaques in the cell culture inoculated with the virus-serum mixture as compared to the control culture (cells incubated with virus alone) indicates the presence of a DENV neutralizing antibody in the serum. The

neutralizing antibody titer is identified as the highest serum dilution that reduces the number of virus plaques in the test by 50% or more.

In addition to PRNT assay, neutralizing antibody can also be measured by an immunostaining-based neutralization assay. The method is identical to the PRNT
5 assay, up to the step of virus-antibody absorption on cell monolayer in a cell culture plate (6-well, 24-well, 48-well, or 96-well plate) at 37°C CO₂ incubator. Instead of Ye-Lah medium with agarose overlay, liquid culture medium or medium mixed with Avicel overlay is added to the plates following the virus absorption. Medium or the Avicel overlay is removed after desired incubation periods (*e.g.* 2-5 days), and cells
10 are fixed with acetone. DENV antibodies are added to the cell plates for 1 hour at 37°C. Plates are washed 3 times to remove unbound antibodies, and chemical or fluorescent conjugated secondary antibody is added to the plate and incubated for 30-60 min at 37°C. The immunostained virus foci in the Avicel overlay plates can be visualized and counted. In the case of using liquid medium in the experiment, the
15 viral antigen produced in the infected cells can be measured by plate reader.

Example 10

Antibody Cross-Reactivity of Chimeric Flaviviruses

This example describes methods for determining reduction of antibody
20 cross-reactivity for DENV E proteins including one or more amino acid substitutions.

DENV E protein (wild type or including one or more amino acid substitutions) is expressed either by infection of cells with a WN/DEN virus chimera encoding a DENV E protein, or by recombinant production of the DENV E proteins
25 (for example by expression in mammalian cells, yeast, or *E. coli*). E protein antigen of the WN/DENV chimera or its variants containing different amino acid substitutions expressed in the chimera-infected C6/36 cells is analyzed with a panel of anti-flavivirus mAbs by IFA to determine mAb end point reactivity of the variant E proteins. Briefly, infected cells are fixed by acetone on microscopy optical slides
30 or slide chambers. Serial diluted mAbs are added to different wells of the slide and incubated at 37°C for 1 hour and unbound mAb is then rinsed away by PBS. Secondary goat- or rabbit-anti-mouse IgG conjugated with FITC is added and

incubated at 37° for 30 min to bind the mouse mAb in the wells, and unbound conjugates is rinsed off by PBS. Positive wells are detected by fluorescent microscope.

Alternatively, purified WN/DENV virus particles are captured in native form
5 by a rabbit anti-DEN polyclonal antibody coated on ELISA plates. The E protein and its variants on the virus particles are analyzed by ELISA with a panel of mouse anti-flavivirus mAbs to determine mAb end point reactivity of the variant viral particles, following the protocol of Roehrig *et al.* (*Virology* 246:317-28, 1998). The panel of the mAb can include 4G2 (ATCC No. HB-112), 6B6C-1, 1B7-5, 1A1D-2,
10 1A5D-1, 1B4C-2, F4540, D1-11, 9F-10, D2811, 2H3, 9A3D-8, 3H5, 1F1, 8A1, and/or 1H10.

WN/DEN chimeras including E protein variants that have reduced antibody cross-reactivity may be used for diagnosis of secondary flavivirus infection with a particular DENV serotype (*i.e.* DEN1, DEN2, DEN3, or DEN4), such as in a PRNT
15 assay. These chimeras may also be included in a flavivirus diagnosis panel, which can reduce false positive results and enhance the speed and accuracy of flavivirus diagnostics.

In view of the many possible embodiments to which the principles of the
20 disclosure may be applied, it should be recognized that the illustrated embodiments are only examples 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.

We claim:

1. A nucleic acid chimera comprising:
a first nucleic acid molecule comprising a 5' non-coding region, a nucleic acid encoding a C protein and non-structural proteins, and a 3' non-coding region from a West Nile virus genome; and
a second nucleic acid molecule operably linked to the first nucleic acid molecule, encoding at least a portion of a prM protein and E protein from a Dengue virus genome.
2. The chimera of claim 1, wherein the West Nile virus genome is a NY99 West Nile virus strain genome.
3. The chimera of claim 1, wherein the Dengue virus genome is a Dengue-1, Dengue-2, Dengue-3, or Dengue-4 genome.
4. The chimera of claim 1, wherein the Dengue virus genome comprises a 16681 Dengue-2 virus strain genome.
5. The chimera of claim 1, wherein the second nucleic acid molecule encodes at least one amino acid substitution in the E protein, wherein the substitution increases virus titer, replication rate, plaque size, or stability in cell culture.
6. The chimera of claim 5, wherein the at least one amino acid substitution in the E protein comprises a substitution at amino acid position 349 of any one of SEQ ID NOs: 2, 4, 6, or 8, position 407 of any one of SEQ ID NOs: 2, 4, 6, or 8, position 471 of any one of SEQ ID NOs: 2, 4, or 8 or 469 of SEQ ID NO: 6, position 488 of any one of SEQ ID NOs: 2, 4, or 8 or 486 of SEQ ID NO: 6, or a combination of two or more thereof.
7. The chimera of claim 6, wherein the at least one amino acid substitution in the E protein is one or more of K122I (K407I in SEQ ID NO: 2), S186F (S471F in SEQ ID NO: 2), and N203D (N488D in SEQ ID NO: 2 or N486D in SEQ ID NO: 6).

8. The chimera of claim 1, wherein the second nucleic acid molecule encodes at least one amino acid substitution in the prM protein, wherein the substitution increases virus titer, replication rate, plaque size, or stability in cell culture.

9. The chimera of claim 1, wherein the first nucleic acid molecule encodes at least one amino acid substitution in one or more of the non-structural proteins or the C protein, wherein the substitution increases virus titer, replication rate, plaque size, or stability in cell culture, or decreases infectivity in mosquitoes.

10. The chimera of claim 9, wherein the amino acid substitution is a substitution selected from the group consisting of position 49 of non-structural protein 2A (NS2A) (position 1181 of SEQ ID NOs: 2, 4, or 8 or position 1179 of SEQ ID NO: 6), position 94 of NS2A (position 1226 of SEQ ID NOs: 2, 4, or 8 or position 1224 of SEQ ID NO: 6), position 241 of non-structural protein 4B (NS4B) (position 2503 of SEQ ID NOs: 2, 4, or 8 or position 2501 of SEQ ID NO: 6), and a combination of two or more thereof.

11. The chimera of claim 10, wherein the amino acid substitution is one or more of NS2A I49T (I1181T of SEQ ID NOs: 2, 4, or 8 or I1179T of SEQ ID NO: 6), NS2A F94L (F1226L of SEQ ID NOs: 2, 4, or 8 or F1224L of SEQ ID NO: 6), and NS4B T241I (T2503I of SEQ ID NOs: 2, 4, or 8 or T2501I of SEQ ID NO: 6).

12. The chimera of claim 1, wherein the first nucleic acid molecule comprises at least one nucleic acid substitution in the 5' non-coding region or the 3' non-coding region of the West Nile virus genome, wherein the substitution increases virus titer, replication rate, plaque size, or stability in cell culture, or decreases infectivity in mosquitoes.

13. The chimera of claim 1, wherein the second nucleic acid molecule encodes at least one amino acid substitution in the E protein, wherein the substituted E-protein exhibits measurably reduced antibody cross-reactivity.

14. The chimera of claim 13, wherein the at least one amino acid substitution in the E protein comprises a substitution at amino acid position 101 (position 386 of any one of SEQ ID NOs: 2, 4, 6, or 8), 106 (position 391 of any one of SEQ ID NOs: 2, 4, 6, or 8), 107 (position 392 of any one of SEQ ID NOs: 2, 4, 6, or 8), 108 (position 393 of any one of SEQ ID NOs: 2, 4, 6, or 8), 135 (position 420 of any one of SEQ ID NOs: 2, 4, 6, or 8), or a combination of two or more thereof.

15. The chimera of claim 1, having a nucleic acid sequence selected from the group consisting of SEQ ID NOs: 1, 3, 5, 7, and nucleic acid sequences at least 95% identical to at least one of SEQ ID NOs: 1, 3, 5, or 7.

16. The chimera of claim 1, having an amino acid sequence selected from the group consisting of SEQ ID NOs: 2, 4, 6, 8, and amino acid sequences at least 95% identical to at least one of SEQ ID NOs: 2, 4, 6, or 8.

17. A method of detecting a Dengue virus neutralizing antibody in a sample from a subject, comprising:

contacting the sample with a virus encoded by the chimera of claim 1 to form a virus-sample mixture;

inoculating a susceptible monolayer cell culture with the virus-sample mixture;

incubating the cell culture under conditions sufficient to allow virus replication;

counting plaques, counting immunostained foci, or measuring viral antigen level in the culture; and

comparing the number of plaques, the number of foci, or the viral antigen level to a control culture, wherein a decrease in the number of plaques, number of foci, or viral antigen level as compared to the control culture indicates the Dengue virus neutralizing antibody is present in the sample.

18. A method of producing virus particles expressing Dengue virus prM, M, and E proteins, comprising:

culturing a virus encoded by the chimera of claim 1 in a cell, wherein virus particles comprising one or more Dengue prM, M, or E proteins are produced;

collecting a supernatant from the cell culture containing the chimera; and
purifying the virus particles from the supernatant.

19. 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 3' non-coding region from a West Nile virus genome;
and

a second nucleic acid molecule operably linked to the first nucleic acid molecule, encoding at least a portion of a C protein, prM protein, and E protein from a Dengue virus genome.

20. A method of detecting a Dengue virus antibody in a sample from a subject, comprising:

contacting the sample with a virus encoded by the chimera of claim 1 under conditions wherein a virus/antibody complex can form; and
detecting formation of the virus/antibody complex.

21. The method of claim 20, wherein detecting formation of the virus/antibody complex comprises:

contacting the sample with a secondary antibody which is reactive with the Dengue virus antibody and which comprises a detectable moiety; and
detecting the detectable moiety.