



(51) International Patent Classification:

A61K 39/12 (2006.01)

(21) International Application Number:

PCT/IB2017/001361

(22) International Filing Date:

29 September 2017 (29.09.2017)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

62/402,640 30 September 2016 (30.09.2016) US
62/524,193 23 June 2017 (23.06.2017) US

(71) Applicant: SANOFI PASTEUR [FR/FR]; 14 Espace Henry Vallée, 69007 Lyon (FR).

(72) Inventors: GIEL-MOLONEY, Maryann; 109 Boyd Street, Watertown, Massachusetts 02472 (US). GIR-ERD-CHAMBAZ, Yves; 22, Chemin de la Pra, 69510 Messimy (FR). GUY, Bruno; 2, rue Casimir Périer, 69002 Lyon (FR). LECOUTURIER, Valérie; 16, rue des Bruyères, 69380 Chazay D'azergues (FR). OOMEN, Ray; 8 Craigie Circle # 27, Cambridge, Massachusetts 02138 (US). PUGACHEV, Konstantin; 566 Commonwealth Avenue Unit 809, Boston, Massachusetts 02215 (US). GONCALVEZ, Ana; 41 Hillside Road, Watertown, Massachusetts 02472 (US). DELAGRAVE, Simon; c/o Sanofi Pasteur Cambridge Campus, 38 Sidney Street, Cambridge, MA 02139 (US).

(74) Agent: ERNEST GUTMANN - YVES PLASSERAUD SAS; 88, Bd des Belges - CS 60151, 69452 Lyon Cedex 06 (FR).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

- with international search report (Art. 21(3))
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))
- with sequence listing part of description (Rule 5.2(a))

(54) Title: LIVE ATTENUATED CHIMERIC ZIKA VIRUS AND ITS USE AS AN IMMUNOGENIC COMPOSITION

(57) Abstract: The present invention is directed to a live attenuated chimeric Zika virus, and its use with a view to generating a neutralising immune response in animals, preferably in humans. The present invention is thus specifically directed to a live attenuated chimeric Zika virus, comprising a yellow fever virus (YFV) genome whose prM-E sequences have been substituted with sequences coding for the membrane precursor (prM) and envelope (E) proteins of a Zika virus (ZIKV). The present invention further relates to an immunogenic composition comprising a live attenuated chimeric Zika virus as defined herein and a pharmaceutically acceptable carrier, as well as said composition for use in medicine. Additionally, the present invention relates to a method of inducing a neutralizing antibody response against Zika virus in a subject, and to a method for producing a live attenuated chimeric Zika virus as defined herein.



LIVE ATTENUATED CHIMERIC ZIKA VIRUS AND ITS USE AS AN IMMUNOGENIC COMPOSITION

REFERENCE TO A SEQUENCE LISTING

The application incorporates nucleotide and/or amino acid sequences which are present in the file named "B12091B SQL_ST25.txt"; this sequence listing is part of the present application.

FIELD OF THE INVENTION

The present invention relates to live attenuated chimeric Zika virus, immunogenic compositions comprising the virus, and uses therefore in a method for inducing a neutralizing immune response against Zika virus in a subject, comprising administering said virus or said immunogenic composition to said subject.

BACKGROUND:

Zika virus (ZIKV) is a member of the *Flavivirus* genus of the *Flaviviridae* family that includes several major human and veterinary pathogens such as yellow fever (YF), dengue types 1-4 (DEN1-4), Japanese encephalitis (JE), West Nile (WN) and tick-borne encephalitis (TBE) viruses. They are maintained in nature in cycles that involve a variety of avian and/or mammalian hosts and transmitting mosquito or tick vectors. Flaviviruses are small (50 nm) enveloped plus-strand RNA viruses. The viral particle contains a nucleocapsid composed of viral RNA and capsid protein C, which is surrounded by a lipid envelope containing the envelope glycoprotein E and membrane protein M.

The genomic RNA of flaviviruses is of approximately 11,000 nucleotides in length and encodes a single open reading frame (ORF) flanked by 5' and 3' untranslated regions (UTRs) of about 120 and 500 nucleotides in length, respectively. The ORF is translated into a polyprotein precursor, in which the three structural proteins (capsid (C), membrane (prM/M, wherein prM is a glycoprotein precursor for mature unglycosylated M) and envelope (E) proteins) are followed by the seven nonstructural proteins NS1 - NS5 involved in virus replication. This structure is common to all flaviviruses. The polyprotein is processed by a combination of cellular and viral (NS2B/NS3) proteases to yield individual proteins. The membrane (M) and envelope (E) proteins form the external surface of the mature virus particle (90 E protein homodimers cover most of the surface), while the uncleaved precursor of M, prM, is found as a prM-E complex on the immature virion.

Flavivirus replication occurs in the cytoplasm of infected cells. Upon infection of cells and translation of genomic RNA, processing of the polyprotein starts with translocation of the prM portion of the polyprotein into the lumen of the endoplasmic reticulum (ER) of infected cells,

followed by translocation of E and NS1 portions, as directed by the hydrophobic signals for the prM, E and NS1 proteins.

The E protein is the main immunogen eliciting neutralizing antibodies that are considered to be the main correlate of immunity against flavivirus infection. Virus-specific cytotoxic T-lymphocyte (CTL) response is the other key attribute of immunity. Multiple CD8+ and CD4+ CTL epitopes have been characterized in various flavivirus structural and non-structural proteins (Gubber et al, 2007; Lindenbach et al, 2007).

Zika virus is a mosquito-borne flavivirus transmitted by *Aedes* mosquitoes. Genetically it belongs to the Spondweni group which is distinct from viruses in the four main flavivirus serocomplexes (YF, JE, dengue and TBE). It was first isolated in Zika Valley, Uganda in 1947 from a febrile Rhesus monkey and until recently, was prevalent only in Africa and Asia. Prior to 2007, its circulation in tropical regions of Africa and Asia resulted in only a few described cases of mild disease in humans. Clinical manifestations resulting from infection with this virus are similar to those that can be caused by some other flaviviruses, especially dengue viruses, and include fever, malaise, headache, dizziness, anorexia, retro-orbital pain, and maculopapular skin rash.

A recent spread of the virus to the Pacific and the Americas has been accompanied by an unprecedented increase in transmission rates and has linked Zika infection to microcephaly (MC) and other birth defects in infants of mothers infected during pregnancy as well as neurological disorders in adults, mostly Guillain-Barré syndrome (GBS). In addition to transmission by mosquitoes which accounts for the vast majority of infections, cases of sexual transmission have been documented. The first Zika outbreak outside of Africa and Asia occurred in 2007 in Micronesia, with 919 residents of Yap Island (18% of the entire population) showing mild symptoms such as rash, fever, myalgia, headache and conjunctivitis. This was followed by an outbreak in French Polynesia in 2013-2014 with around 8,750 cases of symptomatic infection, including 42 cases of GBS (encephalitis, meningitis, paraesthesia, facial paralysis and myelitis). In May 2015, Brazil reported its first Zika case and the disease has subsequently spread to other parts of Brazil and other Latin American countries. In November 2015, a public health emergency was declared in Brazil in relation to an unusual increase in the number of children born with MC attributed to Zika infection of pregnant women. By January 2016, Brazil had an estimated 1.3M cases of Zika and approximately 3,530 suspected MC cases including 46 deaths in 20 states (Fauci et al, 2016). In the continental US, the first cases of local transmission by mosquitoes were reported in July 2016 in Miami, Florida.

Different strategies have been previously used, with more or less success, in vaccination against flaviviruses. Traditional, first-generation flavivirus vaccines for humans are live attenuated vaccines (LAV) obtained by empirical attenuation of wild type viruses (17D vaccine

against YF and the Chinese SA14-14-2 vaccine against JE) and inactivated virus vaccines (INV) available for JE and TBE.

INVs require several doses for initial immunization, followed by periodic boosters to maintain immunity, while the main advantage of LAVs is that they generally require very few doses to elicit durable protective immunity. For example, YF 17D is considered one of the strongest immunogens ever developed in man, providing protection after a single dose that is believed to be life-long. Sanofi Pasteur has more recently developed a novel chimeric LAV platform, ChimeriVax, targeting dengue, JE and WN. ChimeriVax-JE and –DEN vaccines are now registered products licensed as IMOJEV™ and Dengvaxia®, respectively. ChimeriVax vaccines are constructed by replacing the prM-E envelope protein genes in the YF 17D genome with their counterparts from heterologous target flavivirus.

The ChimeriVax® technology indeed essentially relies on the attenuation of a live chimeric virus in such a way that said virus becomes asymptomatic when inoculated, however without losing its capacity to generate a neutralizing immune response.

Some of the flaviviruses appear to be inappropriate for vaccination using the ChimeriVax technology. Indeed, this technology is based on the use of live attenuated viruses, and uncertainty remains about whether the right balance of attenuation and immunogenicity can be achieved, as reported in Rumyantsev et al, 2013. In this article, it was reported that a chimera between the Dengue virus and an attenuated virus serologically related to the Tick-borne encephalitis was *in fine* found over-attenuated, such that no vaccination was possible. For other viruses, the infecting doses are too close to the lethal doses such that no attenuated virus capable of generating a protecting immune response could be obtained.

Alternative technologies, such as RepliVax have thus been developed. RepliVax is an approach available to flavivirus as well as non-flavivirus vaccines based on flavivirus vectors defective in replication due to specifically engineered deletion(s), referred to as pseudo infectious viruses (PIVs). There are two versions of the technology, the single-component PIV approach (sPIV) produced in complementing packaging cell substrates, and two-component PIV approach (tcPIV) (Rumyantsev et al, 2011). RepliVax vaccines have the potential to be safe, in particular due to their single-cycle replication in vivo, while retaining the ability to induce robust and durable immunity after a single dose without adjuvant. This approach has been applied to a “difficult” flavivirus target, namely Tick-borne encephalitis TBE (Rumyantsev et al, 2013, mentioned above) and several non-flavivirus targets such as Rabies, HIV, etc., yielding promising vaccine candidates.

Moreover, because of the concerns related to Zika replication during pregnancy, it could be preferable to use inactivated, adjuvanted virus rather than an attenuated virus.

For rapidly emerging viruses requiring the speedy development of a vaccine, such as Zika, cross-immunity is also an avenue of research. For example, people exposed to another flavivirus, for example Dengue, may also have some immunity to Zika.

In parallel, other approaches have been investigated, namely gene-based vaccination which has led to protective immune responses against another related flavivirus, West Nile virus, both in human studies and in animals. Because DNA and, more recently, RNA vaccines have shown promise and can be relatively readily produced, they offer viable alternatives.

There are thus several different areas of research, with a view to rapidly developing a vaccine and/or antiviral medicines, to prevent and/or treat the infection by Zika virus.

To date, there is no specific treatment for Zika infection.

Since the currently available Zika prevention measures, such as mosquito control and personal protection from bites are limited in efficacy, difficult to enforce and expensive, as already noted for Dengue, a safe and efficacious Zika vaccine would be the best means of prevention. As there is no licensed vaccine currently available, and in view of the risk of pandemic, it is highly desirable to develop an immunogenic composition that is able to induce a neutralising immune response against the Zika Virus in a subject.

SUMMARY OF THE INVENTION

The present invention is directed to a live attenuated chimeric Zika virus, and its use with a view to generating a neutralising immune response against the Zika virus in animals, preferably in humans.

The present invention is thus specifically directed to a live attenuated chimeric Zika virus, comprising a yellow fever virus (YFV) genome whose prM-E sequences have been substituted with sequences coding for the membrane precursor (prM) and envelope (E) proteins of a Zika virus (ZIKV).

The present invention further relates to an immunogenic composition comprising a live attenuated chimeric Zika virus as defined herein and a pharmaceutically acceptable carrier, as well as said composition for use in medicine.

Additionally, the present invention relates to a method of inducing a neutralizing antibody response against Zika virus in a subject, preferably a human subject, wherein said method comprises administering to the human subject the immunogenic composition as defined herein.

The invention further relates to a method for producing a live attenuated chimeric Zika virus as defined herein, said method comprising:

- a) infecting mammalian cells or mosquito cells with a live attenuated chimeric Zika virus as defined herein, or transfecting mammalian cells or mosquito cells with DNA or RNA corresponding to the genome of a said chimeric Zika virus;
- b) cultivating the infected cells in a suitable medium to propagate the live attenuated chimeric Zika virus; and
- c) harvesting the live attenuated chimeric Zika virus.

DESCRIPTION OF THE FIGURES:**Fig.1:** ChimeriVax-Zika designs.

This figure illustrates the designs of some of the ChimeriVax-Zika (CYZ) tested by the inventors.

In the top level, the parent design, corresponding to p391, is illustrated. In this construction, the genes coding for the capsid protein (C) and the non-structural proteins are those of the Yellow Fever 17D (YF 17D) strain and the prM and E sequences are those from the French Polynesia 2013 strain (FP2013, GenBank accession number KJ776791.1). At the junction between the capsid gene (from YF 17D) and the prM gene (from FP2013), the genomic sequence encodes a signal sequence (SS) which is the signal sequence from YF17D. The asterisk means that a silent A to T nucleotide change at nucleotide 166 (Gly codon of the YF 17D C protein amino acid 15) is present within the capsid sequence of YF17D. This change was reverted in an additional variant p411.

The different alternative designs tested by the inventors are illustrated below; only the portion(s) of the sequences which differ(s) from the parent design p391 are illustrated. The sequences not illustrated are identical to those of p391.

Fig. 2. CYZ p391 recovery and replication in Vero cells in serum free medium.

Fig. 2a illustrates Vero cells, 5 days after infection with p391 variant (p391 ChimeriVax French Polynesia, designated p391 CV-FP), at different concentrations, stained by crystal violet.

Fig. 2b illustrates Vero cells infected with p391 virus, methanol – fixed, and stained by immunohistochemistry using monoclonal antibodies specific for the E protein of Zika virus.

Fig. 2c represents the growth curve of the p391 virus in Vero cells in a T25 flask in serum free medium, at a multiplicity of infection (MOI) ranging from 0.001 to 0.1 Plaque Forming Units (PFU)/cell. The x-axis represents the days post infection, and the y-axis represents the pfu/mL.

Fig. 3. Increased replication of p393 CYZ variant with hybrid (YF/ZIKV) signal sequence for prM in Vero cells in serum free conditions.

Fig. 3a illustrates Vero cells, 5 days after infection with p393 virus (passage P1) at different concentrations, stained by crystal violet.

Fig. 3b illustrates Vero cells, infected by the p393 virus, methanol-fixed, and stained by immunohistochemistry using monoclonal antibodies specific for the E protein of Zika virus.

Fig. 3c represents the growth curve of the p393 virus in Vero cells in T25 flask, in serum free medium, at a multiplicity of infection (MOI) ranging from 0.001 to 0.1 PFU/cell. The x-axis represents the days post infection, and the y-axis represents the pfu/mL.

Fig. 4. CYZ p429 variant with chimeric SS and the E-N154Q mutation (the E glycosylation site removed). Data for virus preparations from two transfections using two plasmid clones are shown.

Fig. 4a illustrates Vero cells, 5 days after infection by the p429 virus (from two plasmid clones) at different concentrations, stained by crystal violet.

Fig. 4b represents the results of a plaque assay using methyl cellulose and represents the viral titers of virus grown on Vero cells, in serum free medium, 5 days post transfection (passage P0) or post-infection (passages P1 and P2) by p429. The viral titer resulting from p429-1 plasmid clone is on the right and the viral titer resulting from p429-2 is on the left.

Fig. 5. CYZ p428 variant with chimeric SS and WN04-specific attenuating deletion C2 (PSR) in the YF 17D capsid C protein. Data for virus preparations from two transfections using two plasmid clones are shown.

Fig. 5a illustrates Vero cells, 5 days after infection by the p428 virus (from two plasmid clones) at different concentrations, stained by crystal violet.

Fig. 5b represents the results of a plaque assay using methyl cellulose and represents the viral titers of virus grown on Vero cells, in serum free medium, 5 days post transfection (passage P0) or post-infection (passages P1 and P2) by p428. The viral titer resulting from p428-1 is on the right and the viral titer resulting from p428-2 is on the left.

Fig. 6. Suckling mouse neurovirulence test: ChimeriVax Zika is highly attenuated compared to the YF 17D benchmark and similar to ChimeriVax-JE.

The different graphs illustrate the percentage of survival, from day 0 to day 22 post inoculation of various doses of p391, p393, p411, wt Zika Puerto-Rico (PR) 2015, control YF vaccine (licensed YF-VAX®) and chimeric Japanese Encephalitis virus, respectively. The different inoculated doses shown on the graphs are according to back-titration of inoculates.

Fig. 7. CYZ p393 and wt ZIKV PR 2015 titration by RT-qPCR in tissues of AG129 mice on days 12 - 20 (euthanasia) and A129 mice on day 26 (end of study).

Fig.8. Replication of Zika viruses in human neuroblastoma cells.

The two graphs illustrate the virus growth curves (titers on indicated days post-infection) for different wt ZIKV and CYZ variants, following infection at a MOI of 0.01 (left graph) and 0.001 (right graph).

Fig. 9. CYZ is attenuated in young A129 mice (Experiment 2 of example 4, in A129 mice).

The two graphs illustrate the body weight (A) and survival (B) after subcutaneous inoculation of CYZ p393, wt ZIKV PR-2015 and YF 17D.

A: weight loss of A129 mice immunized with CYZ. B: Survival of A129 mice immunized with CYZ.

Fig. 10: Significant attenuation of CYZ compared to wt ZIKV Puerto-Rico 2015, based on biodistribution in 8 week-old A129 mice on days 5 and 26 post-inoculation (experiment 3 of Example 4, in A129 mice). The two graphs illustrate the RNA viral loads in the spleen, liver, brain and testicles, on days 5 (left panel) and 26 (right panel). The RNA load is expressed in genome equivalents per ml (GE/mL); 1st bar corresponds to CYZ p393 and 2nd bar to wt ZIKV PR.

Fig. 11: CYZ immunized A129 mice are protected from challenge with wt ZIKV.

The graph illustrates the RNA viral loads of ICR mice immunized with different CYZ constructs and then challenged with wt ZIKV.

Fig.12: CYZ vaccine candidates and YF 17D control are highly attenuated compared to wt ZIKV viruses for replication in vitro in human neuronal progenitors cells (NPCs).

The graph illustrates the virus growth curves (titers on indicated days post-infection) for different wt ZIKV and CYZ variants, following infection at a MOI of 0.01.

Fig.13: CYZ vaccine candidates are more attenuated compared to wild type Zika PR strain control in immunocompetent suckling mice.

The graphs report the numbers of sick and healthy animals in the groups receiving the p393 (top) and the wild type ZIKV PR (bottom) viruses.

Definitions:

The term "Zika disease", as used herein, refers to the clinical symptoms, of all grades of severity, exhibited by an individual following infection by a Zika virus. As used herein, the term Zika disease encompasses both the milder manifestations of Zika disease such as a mild febrile illness characterized by fever, rash, arthralgia, myalgia, headache and conjunctivitis, and the more severe manifestations or serious complications of Zika infection, such as the neurological autoimmune disorder Guillain-Barré syndrome and microcephaly in the infants of mothers infected during pregnancy.

The terms "Zika Virus" and "ZIKV" are used interchangeably. They refer to positive single-stranded RNA viruses belonging to the Flavivirus genus of the flaviviridae, which are specifically recognized, as virus, by anti-Zika antibodies, especially by monoclonal antibodies to Zika envelope protein, and more especially by the monoclonal antibodies to Zika envelope protein from Aalto Bio Reagents, Ltd, *inter alia* clones 0402166, 0302156, 0502176, 0602186 or which are identified as a Zika virus by sequencing and alignment with other known Zika sequences. A recombinant chimeric Zika virus, expressing the membrane (M) and envelope (E) proteins of a

ZIKV, but comprising a chimeric viral RNA, for example including sequences from another flavivirus, is thus a Zika virus according to the present invention, provided the inserted prM-E sequence is identified as a Zika sequence by sequence alignment or if the chimeric Zika virus is recognized by anti-Zika antibodies, for example anti-Zika antibodies as defined above; especially, it is recognized, from a serological point of view, as a Zika virus by an infected host. Wild type Zika viruses refer to strains isolated from the natural environment (for example strains isolated from infected persons or mosquitoes) and characterized, by antibodies, as ZIKV. Wild type ZIKV which can be cited include the French Polynesia 2013 strain having GenBank accession number KJ776791.1; the Brazil 2015 strains having GenBank accession numbers KU365779.1, KU365777.1, KU365780.1, KU527068.1 and KU365778.1, the Puerto-Rico 2015 (PR) strain, having GenBank accession number KU501215.1, the French West Indies 2015 strain, having GenBank accession number KU647676.1, the Haiti 2014 strain, having GenBank accession number KU509998.3, and the Cambodia 2010 strain, having accession number JN860885.1.

The term "live attenuated Zika virus", as used herein, refers to a live Zika virus, derived from a wild type Zika virus, by genetic modification resulting in attenuation of virulence and an inability to induce a disease state characterized by the same set of symptoms associated with the corresponding wild type Zika virus. A live attenuated Zika virus may be prepared from a wild type virus, for example, by recombinant nucleic acid technology, site directed mutagenesis, serial passages on replication competent cells, chemical mutagenesis, electromagnetic radiation or genetic manipulation such as the deletion of a small section of the viral nucleic acid. In the context of the invention, "Zika chimera" or "chimeric Zika virus" means a recipient flavivirus in which the genetic backbone of a recipient virus has been modified by exchanging the sequence of at least the E protein of the recipient flavivirus by the corresponding sequence of a Zika virus. Alternatively, and more preferably, the genetic backbone of the recipient flavivirus is modified by exchanging the nucleic acid sequences encoding both the prM and E proteins of the recipient flavivirus by the corresponding sequences of a Zika virus. Typically, the recipient flavivirus may be attenuated. The recipient flavivirus may be a yellow fever (YF) virus, in which case, the chimera is referred to herein as a "chimeric YF/Zika virus" or as a "Chimerivax Zika virus". Preferably, the YF backbone of a chimeric YF/Zika virus according to the present invention is from an attenuated YF virus. They are referred to herein as a "Chimeric Zika Virus", CYZ or CV-Zika.

In one embodiment, the chimeric YF/Zika virus comprises the genomic backbone of the attenuated yellow fever virus strain YF17D (Theiler M. and Smith H.H., 1937). Examples of other attenuated YF strains which may be used include YF17D204 (YF-VAX^(R), Sanofi-Pasteur, Swiftwater, PA, USA; Stamaryl^(R), Sanofi-Pasteur, Marcy l'Etoile, France; ARILVAX^(TM), Chiron, Speke, Liverpool, UK; FLAVIMUN^(R), Berna Biotech, Bern, Switzerland; YF17D-204 France (X15067, X15062)); YF17D-204 (Rice et al., 1985), or the related strains YF17DD (GenBank

accession number U17066), YF17D-213 (GenBank accession number U17067) and the strains YF17DD described by Galler et al, 1998. Advantageously, the recipient flavivirus of a live attenuated chimeric YF/Zika virus of the present invention is YF 17D or YF 17D204.

The ability of a virus or composition of the present invention to provoke an immune response in a subject (i.e. induce the production of neutralizing antibodies) can be assessed, for example, by measuring the neutralizing antibody titer raised against the chimeric Zika virus comprised within the composition. The neutralizing antibody titer may be measured by the Plaque Reduction Neutralization Test (PRNT₅₀) test (Timiryasova, T.M. et al; 2013). Briefly, neutralizing antibody titer is measured in sera collected from subjects to be tested for their level of Zika neutralizing antibodies. If the subject is a vaccinated subject, a sample is collected from said subject at least 28 days following administration of a virus or composition of the present invention. Serial, two-fold (or other, e.g., ten-fold) dilutions of the sera (previously heat-inactivated) are mixed with a constant challenge dose of Zika virus (expressed as PFU/mL). The mixtures are then inoculated into wells of a microplate with confluent Vero cell monolayers. After adsorption, cell monolayers are incubated for a few days. The presence of Zika virus infected cells is indicated by the formation of infected foci (i.e. plaques) and a reduction in virus infectivity due to the presence of neutralizing antibodies in the serum samples (i.e. a reduction in the number of plaques) can thus be detected. The reported value (end point neutralization titer) represents the highest dilution of serum at which ≥ 50 % of Zika challenge virus (in plaque counts) is neutralized when compared to the mean viral plaque count in the negative control wells (which represents the 100% virus load). The end point neutralization titers are presented as continuous values. The lower limit of quantification (LLOQ) of the assay is 10 (1/dil). It has been commonly considered that seroconversion occurs when the titer is superior or equal to 10 (1/dil). As PRNT tests may slightly vary from a laboratory to another the LLOQ may also slightly vary. Accordingly, in a general manner, it is considered that seroconversion occurs when the titer is superior or equal to the LLOQ of the test.

The term "CCID₅₀" refers to the quantity of virus (e.g. vaccinal virus) infecting 50% of the cell culture. The CCID₅₀ assay is a limit dilution assay with statistical titer calculation.

As used herein, a "Zika naïve" subject refers to a subject who has not been infected by a Zika virus nor previously immunized with a Zika vaccine, i.e. a serum sample taken from said subject will produce a negative result in a Zika ELISA or PRNT₅₀ assay. In respect of the PRNT₅₀ assay, a serum sample from a "Zika naïve" subject will produce a result below the LLOQ of the assay.

As used herein, a "Zika immune" subject refers to a subject who has been infected by a Zika virus or immunized by a Zika vaccine before administration of the virus or composition of the present invention, i.e. a serum sample taken from said subject will produce a positive result in a Zika ELISA or PRNT₅₀ assay. In respect of the PRNT₅₀ assay, a serum sample from a "Zika immune" subject will produce a result above the LLOQ of the assay.

In accordance with the present invention, an “attenuating mutation” refers to a mutation, within a flavivirus or chimeric flavivirus, which is associated with an attenuated form of the flavivirus or chimeric flavivirus, compared to the same flavivirus or chimeric flavivirus without the mutation. Such attenuating mutations are for example the mutations at positions 107, 138, 176 and 280 in the attenuated strain SA14-14-2 of Japanese encephalitis (JE) with respect to the wild type JE virus, as well as the mutations at positions 316 and 440 of said strain (Arroyo et al, 2004).

As used in the present description, by the DNA sequence “equivalent” to an RNA sequence, it is meant the DNA sequence corresponding to the RNA sequence wherein the uridine nucleotides have been replaced by deoxythymidine nucleotides. Similarly, by the RNA sequence “equivalent” to a DNA sequence, it is meant the RNA sequence corresponding to the DNA sequence wherein the deoxythymidine nucleotides have been replaced by uridine nucleotides. The RNA sequence equivalent to a DNA sequence is thus the RNA obtained by transcription of the complementary strand of the DNA.

DETAILED DESCRIPTION OF THE INVENTION:

The present inventors have designed a live chimeric Zika virus, which is simultaneously attenuated, namely has an attenuated phenotype with respect to wild type Zika, which is not neurovirulent, which is genetically stable and safe, which exhibits satisfying manufacturability, and is sufficiently immunogenic to induce the production of neutralizing antibodies, which are effective against Zika strains of both American/Asian origin and African origin.

The invention is more specifically directed to a live attenuated chimeric Zika virus, which comprises essentially the viral genome of a yellow fever virus (YFV), referred to as the YFV backbone in the following, in which the prM-E sequences of YFV have been replaced by heterologous sequences, namely heterologous prM-E sequences, coding for the membrane precursor (prM) and envelope (E) proteins of a Zika virus (ZIKV).

The RNA viral genome of the chimeric virus according to the invention thus comprises sequences coding for the capsid protein and the nonstructural proteins of YFV and sequences coding for the prM and E proteins of a Zika virus. According to the invention, prM and E proteins of a Zika virus are proteins the sequence of which is specific to ZIKV and distinguishable (for example by sequence alignment) from prM and E protein sequences of other viruses, especially of other flaviviruses. Moreover, membrane and envelope proteins of a Zika virus are specifically recognized by anti-Zika antibodies, for example monoclonal anti-Zika antibodies from Aalto Bio Reagents Ltd as referred to above.

It is to be noted that a live attenuated chimeric Zika virus according to the invention thus exhibits, at its surface, membrane and envelope proteins which are ZIKV specific; for the host, the live attenuated chimeric Zika virus of the invention is thus serologically a Zika virus.

The chimeric Zika virus of the invention is a live virus; it is capable of infecting permissive cells. The chimeric virus is moreover able to replicate within infected cells, producing additional

chimeric Zika viruses. This means that the viral genome as designed, once transfected into the cytoplasm of an infected cell, is translated into a long polyprotein, is efficiently processed, the viral genome is replicated and viral particles comprising the viral genome are produced. A complete viral life cycle is obtained after transfection of permissive cells by the chimeric Zika virus according to the invention.

Such permissive cells, which can be infected and allow replication of the virus, are *inter alia* human cells, as well as other mammalian cells such as Vero cells, for example Vero cells ATCC CCL-81. Alternative suitable cells are BHK-21 (for example ATCC CCL-10), C7/10 cells, LLC-MK2, FRhL or MRC-5 cells. Such cells can be used to produce the virus in high quantities, especially with a view to producing vaccines.

The inventors have moreover shown, as illustrated in the experimental section, that a chimeric virus of the invention is genetically stable; no mutation has indeed been observed after several passages. Such a feature is highly desirable with a view to designing a vaccine.

As mentioned above, the chimeric Zika virus is moreover attenuated with respect to wild type Zika virus. This feature is *inter alia* demonstrated in the experimental section. It is preferably highly attenuated with respect to wild type Zika viruses. The chimeric virus according to the invention is not neurovirulent, although the YFV is neurovirulent, this feature is not transferred to the chimeric virus. The design of the YFV/ZIKV chimeric virus of the invention thus provides a virus which is less virulent than both YFV and ZIKV.

The inventors have also shown in a model specifically adapted for determining the localization of the virus in an infected host that the chimeric Zika virus does not replicate efficiently in testes, and does not persist in testes. This is an important safety feature, in view of the recent reports of sexual transmission of wt ZIKV.

According to a preferred embodiment, the genomic viral RNA of the chimeric Zika virus of the invention comprises a yellow fever virus (YFV) genome, except for the part of the genome corresponding to the prM-E sequences, which are the corresponding sequences of a ZIKV. The prM-E sequences of a ZIKV are to be understood according to the invention as sequences coding for a prM protein and E protein specific to ZIKV and thus *inter alia* recognized by anti-Zika antibodies. Sequences specific to ZIKV can also be recognized phylogenetically by sequence alignment. It is stressed in this respect that the genome organization is similar for all flaviviruses, such that the prM-E sequences of a given flavivirus are readily identifiable by a skilled person. Moreover, ZIKV can be distinguished from other species of flaviviruses by its sequence.

Preferably, the ZIKV sequences are integrated in the yellow fever virus (YFV) genome precisely at the positions of the naturally residing prM/E sequences of the yellow fever virus, therefore completely replacing the naturally residing homologous prM/E sequences of the yellow fever virus. A chimeric Zika virus of the invention thus has a chimeric genome, comprising both YFV sequences and ZIKV sequences.

According to a preferred embodiment, the heterologous prM-E sequences of a live attenuated chimeric Zika virus according to the invention, code for a prM and E proteins derived from a specific Zika strain, namely the Zika virus French Polynesia 2013 strain (FP2013). The prM and E proteins of FP2013 have the sequences SEQ ID NO: 3 and 4 respectively, corresponding collectively to SEQ ID NO: 2.

The prM/E amino acid sequence of this strain is identical to the Brazilian 2015 strains, which is important for vaccination against the more recent strains; moreover, the use of the sequences characteristic of this strain has allowed the most advantageous attenuation in the chimeric Zika virus design elaborated by the inventors. Additionally, the inventors have provided evidence that such a chimeric Zika virus elicits neutralizing antibodies against strains of different origins.

The invention is more specifically directed to a chimeric Zika virus, wherein the heterologous prM-E sequences coding for the prM and E proteins, are derived from the Zika virus French Polynesia 2013 strain (FP2013).

The prM-E sequence of the chimeric virus thus comprises or consists of the RNA sequence set forth in SEQ ID NO: 1 or a sequence having at least 99% sequence identity with SEQ ID NO: 1. SEQ ID NO: 1 corresponds to the prM/E sequence of a wild type Zika virus, namely to the prM/E sequence of the FP2013 strain. The prM/E sequence set forth in SEQ ID NO: 1 codes for the polypeptide having the amino acid sequence SEQ ID NO: 2, consisting of the prM and E proteins of French Polynesia 2013 strain.

Methods for defining the sequence identity between two nucleotide sequences, either DNA or RNA, are well known to the skilled person. Suitable programs include for example the Clustal W and Clustal X multiple sequence alignment programs (Larkin et al, 2007) and the MAFFT program (Kato et al, 2002).

In a preferred embodiment, the prM-E sequence of a live attenuated chimeric Zika virus according to the invention comprises or consists of a sequence having at least 99.2% sequence identity to SEQ ID NO: 1, preferably at least 99.4% or 99.5% sequence identity. In most preferred embodiments, the sequence identity is at least 99.6% or at least 99.7%, preferably at least 99.8% or even at least 99.9% sequence identity. The prM-E sequence has preferably the same length as SEQ ID NO: 1, namely 2016 nucleotides, or between 2006 and 2026 nucleotides, preferably between 2012 and 2020 nucleotides.

According to another distinct embodiment, the prM-E sequence of a virus of the invention has less than 99% sequence identity to SEQ ID NO: 1, provided that such a sequence codes for a protein having the sequence SEQ ID NO: 2, namely the prM-E sequence codes for the prM and E proteins of ZIKV FP2013, but differs from SEQ ID NO: 1 due to degeneracy of the genetic code.

According to another embodiment, the viral genome of a chimeric Zika virus of the invention codes for an envelope (E) protein comprising or consisting of the sequence SEQ ID NO: 4,

corresponding to the E protein sequence of the ZIKV FP2013 strain, or for an envelope protein comprising or consisting of a sequence differing from SEQ ID NO: 4 by 1 to 4 mutations, for example a sequence having 1, 2, 3 or 4 mutations compared to SEQ ID NO: 4. By mutation in an amino acid sequence with respect to a reference sequence, it is to be understood either the deletion of one amino acid in the sequence with respect to the reference sequence, or the insertion of one amino acid, or the substitution of one amino acid by another one with respect to the reference sequence.

Particularly preferred mutations according to the present invention are substitutions. The prM-E sequences of the chimeric Zika virus of the invention thus preferably code for an envelope protein comprising or having the sequence SEQ ID NO: 4 or a sequence having 1, 2, 3 or 4 substitutions with respect to SEQ ID NO: 4.

It is also preferred that said mutation is not at the position corresponding to position 473 of SEQ ID NO: 4, namely methionine at position 473 is not substituted by another amino acid in the E protein of the chimeric Zika virus according to the invention.

The sequence of the E protein of a chimeric Zika virus thus comprises or consists of a sequence comprising from 1 to 4, for example 1, 2, 3 or 4 mutations, preferably substitutions compared to SEQ ID NO: 4 provided the amino acid at the position corresponding to position 473 of SEQ ID NO: 4 is not mutated with respect to SEQ ID NO: 4. It is to be noted in this regard that, if the mutations are deletions or insertions, the position corresponding to position 473 of SEQ ID NO: 4 is not necessarily the 473rd position in the envelope protein of the chimeric Zika virus.

According to still another embodiment of the invention, the viral genome of a chimeric Zika virus of the invention codes for a membrane precursor prM protein comprising or consisting of the sequence SEQ ID NO: 3, corresponding to the prM protein sequence of the ZIKV FP2013 strain, or for a prM protein comprising or consisting of a sequence differing from SEQ ID NO: 3 by 1 to 4 mutations, for example a sequence having 1, 2, 3 or 4 mutations compared to SEQ ID NO: 3, and preferably a sequence having 1 or 2 mutations compared to SEQ ID NO: 3, most preferably only one mutation. The meaning of mutation is as defined above.

Particularly preferred mutations according to the present invention are substitutions. The prM-E sequences of the chimeric Zika virus of the invention thus preferably code for a membrane precursor protein comprising or having the sequence SEQ ID NO: 3 or a sequence having 1 to 4, preferably 1 or 2 substitutions with respect to SEQ ID NO: 3.

In a preferred embodiment, the prM-E sequences of the chimeric Zika virus of the invention code for an envelope protein comprising or consisting of SEQ ID NO: 4 or comprising or consisting of a sequence having from 1 to 4 mutations with respect to SEQ ID NO: 4, and a prM protein comprising or consisting of SEQ ID NO: 3, or comprising or consisting of a sequence having 1 or 2 mutations with respect to SEQ ID NO: 3. In a specific embodiment, the prM-E sequences code for an envelope protein having 2 to 4 substitutions with respect to SEQ ID NO: 4 and a membrane precursor having 1 or 2 substitutions with respect to SEQ ID NO: 3.

In a most preferred embodiment, the prM-E sequences of the chimeric Zika virus of the invention code for an envelope protein having SEQ ID NO: 4 or a sequence having from 1 to 4 mutations with respect to SEQ ID NO: 4, and a prM protein having SEQ ID NO: 3 or a sequence having 1 or 2 mutations with respect to SEQ ID NO: 3. In a specific embodiment, the prM-E sequences code for an envelope protein having 2 to 4 substitutions with respect to SEQ ID NO: 4 and a membrane precursor having 1 or 2 substitutions with respect to SEQ ID NO: 3.

As mentioned above, in the context of the present invention, preferred mutations are substitutions and especially preferred mutations are conservative substitutions. Alternative preferred mutations are attenuating mutations, preferably attenuating substitutions, giving rise to an attenuation of the virulence of the virus.

Attenuating mutations are indeed preferred insofar as they not only achieve a greater attenuation of the virulence of the virus, but they also diminish the risk of reversion to virulence of the attenuated virus.

In this respect, a particularly preferred position for the mutation or substitution mentioned above with respect to the E protein sequence of FP2013 strain or of another strain, is the position corresponding to position 319 of SEQ ID NO: 4. It is thus desirable to introduce a modification in the sequence of the envelope protein of a chimeric Zika virus of the invention, with respect to the envelope protein of FP2013 strain, corresponding to SEQ ID NO: 4, by introducing at least one mutation at the position corresponding to position 319 of SEQ ID NO: 4, such that the amino acid (Alanine) present at this corresponding position be either deleted or substituted by another amino acid. Preferably, the amino acid present at this position is substituted by a Valine (V).

Such a mutation at the position corresponding to position 319 of SEQ ID NO: 4 is indeed known as being an attenuating mutation, from the study of the attenuated JE strain SA14-14-2 and ChimeriVax-WN (corresponding to position 316 in this construct).

Similarly, another preferred position for the mutation or substitution with respect to the envelope protein sequence of FP2013 strain, or of another strain, is the position corresponding to position 443 of SEQ ID NO: 4. It is thus desirable to introduce a modification in the sequence of the E protein of a chimeric Zika virus of the invention, with respect to the envelope protein of the FP2013 strain, corresponding to SEQ ID NO: 4, by introducing at least one mutation at the position corresponding to position 443 of SEQ ID NO: 4, such that the amino acid (Lysine) present at this corresponding position be either deleted or substituted by another amino acid by comparison to SEQ ID NO: 4. Preferably, the amino acid present at this position is substituted by an Arginine (R).

This mutation at the position corresponding to position 443 of SEQ ID NO: 4 is also known as being an attenuating mutation, from the study of the attenuated JE strain SA14-14-2 and ChimeriVax-WN (corresponding to position 440 in this construct).

The chimeric Zika virus of the invention thus preferably comprises two mutations with respect to the sequence of the envelope protein corresponding to SEQ ID NO: 4, namely a mutation at the position corresponding to position 319 and another one at the position corresponding to position 443 of SEQ ID NO: 4, but wherein there is no mutation at the position corresponding to position 107 of SEQ ID NO: 4; preferably it comprises substitutions at the two mutated positions, and more preferably the replacement of the amino acid corresponding to position 319 by a Valine, and the amino acid corresponding to position 443 by an Arginine. As detailed previously, the virus may also comprise additional mutations, namely one or two additional mutations in the (E) protein sequence with respect to SEQ ID NO: 4, possibly in addition to 1, 2, 3 or 4, preferably 1 or 2, mutations in the (prM) protein sequence, with respect to SEQ ID NO: 3.

One further position for the mutation or substitution with respect to the envelope protein sequence of FP2013 strain, or of another strain, is the position corresponding to position 107 of SEQ ID NO: 4. It is thus desirable to introduce a modification in the sequence of the E protein of a chimeric Zika virus of the invention, with respect to the envelope protein of the FP2013 strain, by introducing at least one mutation at the position corresponding to position 107 of SEQ ID NO: 4, such that the Leucine (L) present at this position in SEQ ID NO: 4 be either deleted or substituted by another amino acid. Preferably, the Leucine present at this position is substituted by a Phenylalanine (F).

This mutation is also an attenuating mutation initially identified in the attenuated JE strain SA14-14-2. In a preferred embodiment, this mutation is combined with the two mutations, at the positions corresponding to positions 319 and 443 of SEQ ID NO: 4.

According to another embodiment, the (E) protein sequence of a chimeric Zika virus does not comprise a glycosylation site. If the (E) protein sequence of the chimeric virus is derived from the corresponding sequence of the FP2013 strain, then the position corresponding to position 154 of SEQ ID NO: 4, which is a glycosylation site, is mutated either by deletion of the Asparagine (N) at the position corresponding to position 154 of SEQ ID NO: 4, or by replacement of said Asparagine by another amino acid, and especially by Glutamine (Q). Alternatively, the Asparagine may also be substituted by an Alanine (A) or by a Glutamic Acid (E).

This mutation, at the position corresponding to position 154 of SEQ ID NO: 4 is advantageously combined with the previously described mutations, especially the mutations at the positions corresponding to positions 319 and 443 of SEQ ID NO: 4, and possibly also with the mutation at the position corresponding to position 107 of SEQ ID NO: 4.

According to another embodiment, a preferred position for the mutation or substitution mentioned above with respect to the (M) protein sequence of FP2013 strain, or of another strain, is the position corresponding to position 60 of SEQ ID NO: 5. It is thus desirable to introduce a modification in the sequence of the membrane protein of a chimeric Zika virus of the

invention, with respect to the membrane protein of FP2013 strain, corresponding to SEQ ID NO: 5, by introducing at least one mutation at the position corresponding to position 60 of SEQ ID NO: 5, such that the Lysine present at this position be either deleted or substituted by another amino acid. Preferably, the Lysine present at this position is substituted by a Cysteine (C).

As detailed above, this mutation in the membrane protein of the chimeric Zika virus of the invention can advantageously be combined with the other preferred mutations in the envelope proteins. It is particularly envisaged that the preferred mutation in the membrane protein be combined with at least one preferred mutation in the envelope protein, for example the mutation at the position corresponding to position 319 or 443 of SEQ ID NO: 4, or with both mutations, advantageously with the further mutation at the position corresponding to the glycosylation site of the envelope protein. Accordingly, a particularly preferred chimeric Zika virus has a sequence coding for a membrane protein comprising one mutation in the membrane with respect to SEQ ID NO: 5, namely at position 60, and at least 3 mutations in the envelope with respect to SEQ ID NO: 4, namely at positions 154, 319 and 443.

In designing the chimeric Zika virus of the invention, the inventors have moreover defined modifications to be introduced into the sequence encoding the signal sequence residing between the sequences coding for the capsid and prM proteins, in order to optimize this signal sequence. This sequence is indeed at the interface between the YFV sequences, comprising the sequences encoding the capsid protein, and the prM-E ZIKV sequences. This signal sequence is responsible, once translated, for the efficient cleavage of the polyprotein, and thus for an efficient viral life cycle.

The inventors have now determined that sequences encoding the C-prM signal sequence, in a chimeric Zika virus of the invention, are advantageously sequences coding for a hybrid C-prM signal sequence, namely a hybrid yellow fever–Zika C-prM signal sequence. Such a hybrid yellow fever–Zika C-prM signal sequence indeed gives rise to a better immunogenicity of the virus, preserves the growth performance of the chimeric virus, and preserves the attenuation of the chimeric virus. Whereas other hybrid C-prM signal sequences have already been envisaged previously, namely hybrids of yellow fever virus and tick-borne encephalitis (WO2009/114207), these previous hybrid signal sequences were responsible for an increase in virulence. Unexpectedly, the hybrid YFV/ZIKV C-prM signal sequence does not increase the virulence of the chimeric virus, whilst maintaining the advantageous growth performance and increasing the immunogenicity of the chimeric virus.

According to another aspect of the invention, the live attenuated chimeric Zika virus of the invention advantageously comprises genomic sequences coding for a hybrid yellow fever–Zika C-prM signal sequence. More specifically, the sequences between those encoding the YFV capsid protein and those encoding the ZIKV prM protein are hybrid between YFV sequences and ZIKV sequences. Preferably, these sequences code for a hybrid signal sequence having

the sequence SHDVLTVQFLILGMLLTAMA (SEQ ID NO: 6), wherein the first 16 amino acids correspond to the N-terminal part of the YFV C-prM signal sequence (80%) and the last 4 amino acids correspond to the C-terminal part of the ZIKV C-prM signal sequence (20%). It is noted in this regard that the hybrid YFV/TBE C-prM signal sequences described before the present invention had a different proportion of YFV and TBE sequences.

Alternatively, the sequences between those encoding the YFV capsid protein and those encoding the ZIKV prM protein code for a hybrid signal sequence having one or two mutations, preferably only one mutation, even more preferably one substitution, with respect to SEQ ID NO: 6.

The features in connection with the hybrid C-prM signal sequence can advantageously be combined with the other preferred features of a chimeric Zika virus of the invention. These features are *inter alia* advantageously combined with the preferred prM-E sequences, namely those of the FP2013 strain, or derivatives thereof by one or more mutations, as described in the preceding section, especially those derived by the introduction of attenuating mutation(s). Alternatively, the features in connection with the hybrid C-prM signal sequence can advantageously be combined with the preferred prM-E sequences, namely those of the FP2013 strain, but wherein the resulting chimeric Zika virus comprises no further mutations within the prM-E sequence.

According to another embodiment, the yellow fever virus backbone of the chimeric Zika virus of the invention is derived from an attenuated yellow fever strain, preferably from a highly attenuated strain. Such an attenuated strain is preferably the attenuated yellow fever strain 17D. Indeed, while wt YFV can infect, disseminate and be transmitted by *A. aegypti* mosquitoes, YF 17D may infect epithelial cells of the midgut but does not disseminate and is not transmitted. The use of the YF 17D backbone is thus particularly preferred in the context of the present invention.

According to this aspect, the chimeric Zika virus thus comprises genomic sequences coding for the capsid protein of the YFV-17D; namely comprises genomic sequences coding for the protein having SEQ ID NO: 7. Still according to this aspect, the chimeric virus comprises genomic sequences corresponding to the 3'UTR of YFV-17D. A chimeric Zika virus of the invention thus comprises genomic sequences corresponding to SEQ ID NO: 8. It is preferred that a chimeric virus of the invention comprises genomic sequences corresponding to SEQ ID NO: 8 and sequences coding for SEQ ID NO: 7.

Alternative attenuated yellow fever strains are well known to the skilled person. Suitable alternative YFV strains are *inter alia* YF17D204 or YF17DD.

According to still another aspect of the invention, the inventors have also defined that some additional variations of the yellow fever backbone may improve the properties of the chimeric Zika virus, especially may achieve additional attenuating effects in the already chimeric attenuated virus. The invention is thus also directed to chimeric Zika virus having a YFV

backbone comprising additional variations with respect to WT YFV, especially with respect to YFV-17D, resulting in an equivalent and preferably more attenuated chimeric virus. The invention thus encompasses chimeric Zika virus having a YFV backbone genomic sequence encoding a capsid (C) protein sequence which comprises a 3 amino acid deletion at the positions corresponding to positions 40 to 42 of SEQ ID NO: 7, which is the sequence of the capsid protein of YFV-17D.

Alternatively, the genome of a chimeric Zika virus according to the invention may comprises a 5 nucleic acid deletion in the 3'UTR sequence by reference to the 3'UTR of the YFV-17D backbone. Preferably, the 5 nucleic acid deletion in the 3'UTR sequence is at the position which corresponds to positions 256 to 260 of SEQ ID NO: 8, which is the sequence of the 3'UTR of YFV-17D. The resulting 3'UTR sequence, with the deletion, is set forth in SEQ ID NO: 9.

According to a preferred embodiment, the genome of a chimeric Zika virus comprises both a sequence coding for the SEQ ID NO: 7 with a 3 amino acid deletion at the positions 40-42, and sequences corresponding to SEQ ID NO: 9.

According to another aspect, the present invention is also directed to an immunogenic composition, comprising a live attenuated chimeric Zika virus, as defined above, and a pharmaceutically acceptable carrier or excipient.

The pharmaceutically acceptable carrier may be any carrier or excipient which has been accepted in the manufacture of a medicament. The resulting immunogenic composition according to the invention is a pharmaceutical composition.

The pharmaceutically acceptable carrier or excipient according to the present invention means any solvent or dispersing medium commonly used in the formulation of pharmaceuticals and vaccines to enhance stability, sterility and deliverability of the active agent and which does not produce any adverse event, for example an allergic reaction, in a subject. The excipients or carrier is selected on the basis of the pharmaceutical form chosen, the method and the route of administration. Appropriate excipients, and requirements in relation to pharmaceutical formulation, are well known to the skilled person. Particular examples of pharmaceutically acceptable excipients include water, phosphate-buffered saline (PBS) solutions, a 0.4% saline solution, a 0.9% saline solution and a 0.3% glycine solution.

A vaccine composition may optionally contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like.

The composition may also comprise adjuvant, although live viruses used in vaccination are usually used without need for an adjuvant. However, the composition may for example comprises other adjuvanted antigens. The adjuvants are commonly used as 0.005 to 0.5 percent suspension in PBS. Adjuvants enhance the immunogenicity of an antigen but are not necessarily immunogenic themselves. Adjuvants to be added to the composition of the invention are preferably aluminum oxyhydroxide and/or aluminum hydroxide phosphate

(collectively commonly referred to as alum). Alternative adjuvants which can be cited are salts of calcium, iron or zinc, insoluble suspension of acylated tyrosine, acylated sugars, and muramyl peptides (e.g., N-acetyl-muramyl-L-threonine-D-isoglutamine (thr-MDP), N-acetyl-normuramyl-L-alanyl-D-isoglutamine (norMDP), N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)-ethylamine (MTP-PE), etc.).

If intended to be lyophilized, a composition of the invention may also comprise one or several stabilizing excipients such as a sugar or a sugar alcohol, or a mixture of two or more of these.

By an immunogenic composition, it is to be understood a composition capable of generating a neutralizing immune response in a host having received said composition, preferably in humans, either Zika-immune or Zika-naïve subjects. The immune response is a neutralizing immune response directed to ZIKV antigens, namely directed to the ZIKV membrane and/or envelope proteins of the chimeric Zika virus of the invention. The immune response directed to ZIKV antigens is due to the presence in the composition of the live attenuated chimeric Zika virus, exhibiting membrane and envelope Zika protein at its surface. It is stressed in this respect that the live attenuated chimeric Zika virus of the invention does not raise antibodies against YFV. The immunogenic composition of the invention thus elicits a specific humoral response toward the Zika virus.

The immune response comprises the production of antibodies, specifically of neutralizing antibodies. The immune response preferably allows protection of the host against any future infection by ZIKV.

The immune composition may be in a liquid, dried or lyophilized form. If it is in a dried or lyophilized form, for example powder, it is generally to be re-suspended in a liquid formulation before any use. The immunogenic composition may also be frozen.

The immunogenic composition is preferably for administration to a subject, preferably a mammal subject, even most preferably a human subject. The composition is preferably for use in medicine, preferably for use in vaccinating subjects against Zika virus, especially human subjects. The immunogenic composition is thus preferably a vaccine composition.

Preferred subjects to be vaccinated by the immunogenic composition of the invention are Zika-naïve subjects, especially subjects who do not have but are at risk of being infected by Zika virus. Alternatively, the immunogenic composition may also be administered to subjects who have already been infected by Zika, i.e. Zika-immune subjects.

According to another embodiment, the immunogenic composition is for use in a method of inducing a neutralizing antibody response against Zika virus in a mammal, wherein said method comprises administering said immunogenic composition to said mammal. Said mammal is preferably a human. Said human is preferably flavivirus naïve, or preferably Zika naïve and dengue naïve. Alternatively, the composition may also be administered to human subjects who are Zika naïve but dengue immune, Zika immune but dengue naïve, or who are Zika immune and dengue immune. The definitions of "dengue immune" and "dengue naïve" are identical to those of "Zika immune" and "Zika naïve" but with dengue virus.

Preferred human subjects to be vaccinated are children, who are at least 9 months old or at least 2 years old. Preferably said human subjects are at least 5, 7 or preferably 9 years old. Preferably said human subjects are at least 12 years old. Preferably said human subjects (of both sexes) are aged between 2 and 60 years old, most preferably aged between 9 and 60 years old. Other preferred subjects are very young women not yet in childbearing age, e.g. aged between 9 months and 9 years old. Another group of preferred subjects include reproductive age women, e.g. women aged between 9 and 45 years old.

A vaccine composition according to the present invention may be administered in multiple doses, for example in one, two or three doses.

A vaccine composition according to the present invention may advantageously be administered in a single dose.

Optionally, booster administrations of a vaccine composition according to the present invention may be used, for example between six months and ten years, for example six months, one year, three years, five years or ten years after initial immunization (i.e. after administration of the last dose scheduled in the initial immunization regimen).

A human subject according to the present invention (to which a vaccine composition of the present invention is administered) is preferably resident in or travelling to a Zika endemic area. More preferably, said human subject is resident in a Zika endemic area. A human subject according to the present invention may also be resident in an area that is experiencing a Zika epidemic.

Preferably, the immunogenic composition or vaccine composition of the invention reduces the incidence or likelihood of Zika disease. Preferably, the composition according to the invention results in the prevention of Zika disease caused by Zika virus.

The exact quantity of the live attenuated chimeric Zika virus of the present invention to be administered may vary according to the age and weight of the subject, the frequency of administration and the other ingredients in the composition. Generally, the quantity of live attenuated chimeric Zika virus comprised in a dose of a vaccine composition of the invention lies within a range of from about 10^3 to about 10^6 CCID₅₀, for example in the range of from about 5×10^3 to about 5×10^5 .

An immunogenic composition according to the invention can be packaged in unit dose or in multiple dose form, wherein a dose preferably corresponds to a volume of 0.5 mL. The immunogenic composition may be packaged *inter alia* in ready-filled syringes, in vials, or in any other suitable container.

The invention is also directed to a method of inducing a neutralizing antibody response against Zika virus in a subject, preferably a human subject, comprising administering the immunogenic composition of the invention, or the live attenuated chimeric Zika virus of the invention to the subject. Preferably the antibody response induced by the method is neutralizing in respect of an Asian/American Zika strain (e.g. Puerto Rico-2015) and an African Zika strain (e.g. Uganda MR766). The administration is achieved by any suitable route of administration. Examples of

suitable routes of administration include for instance intramuscular, transcutaneous, subcutaneous, intranasal, oral or intradermal. Advantageously, the route of administration is subcutaneous.

According to still another aspect, the present invention is also directed to a recombinant nucleic acid, corresponding to the chimeric Zika virus of the invention, especially corresponding to the genome of such a virus. Preferably, the recombinant nucleic acid thus corresponds to the YFV backbone, in which heterologous sequences corresponding to prM-E sequences of a Zika virus have been inserted. The YFV backbone is preferably the YFV-17D backbone, as already mentioned and the prM-E sequences are advantageously derived from those of the French Polynesia 2013 strain of Zika virus, as detailed in the preceding sections. The nucleic acid thus comprises or consists in the RNA sequence equivalent to the DNA sequence set forth in SEQ ID NO: 10, or equivalent to the DNA sequence having at least 90 %, preferably at least 95%, most preferably at least 96%, 97%, 98% or even at least 99% sequence identity to said sequence, provided said sequence has at least a subsequence, corresponding to the prM-E sequence, which has at least 99% sequence identity to the portion of SEQ ID NO: 10 corresponding to the prM/E sequences, namely the portion between nucleotide 482 and nucleotide 2497 of SEQ ID NO: 10. The sequence between nucleotide 482 and nucleotide 2497 of SEQ ID NO: 10 is the DNA sequence that is equivalent to the RNA sequence SEQ ID NO: 1. The invention is also directed to the recombinant DNA nucleic acid molecule that is equivalent to this RNA sequence (SEQ ID NO: 1), as well as a nucleic acid molecule comprising such a DNA sequence.

The invention also concerns a vector, comprising the recombinant DNA or RNA nucleic acid as defined above, preferably comprising the recombinant DNA nucleic acid. The recombinant DNA nucleic acid is preferably operably linked to a promoter in a vector as defined; preferably, the promoter is linked in such a manner that it allows the transcription of the strand of the DNA generating a RNA nucleic acid corresponding to the RNA recombinant nucleic acid as defined above. Such a RNA molecule corresponds to the viral genome of a chimeric Zika virus according to the present invention. A preferred promoter is a eukaryotic promoter.

The vector may be any type of vector. Preferred vectors are for example plasmids or phages, which can be replicated within cells, especially within bacterial cells or eukaryotic cells, using the cellular machinery.

According to still another aspect, the invention is also directed to a method for producing a live attenuated chimeric Zika virus as defined, wherein the method comprises the following steps:

- a) infecting mammalian cells or mosquito cells with a live attenuated chimeric Zika virus of the invention, or transfecting the cells with either a RNA recombinant nucleic acid as defined above, or with a DNA vector as defined above, preferably containing a eukaryotic promoter;

- b) cultivating the infected cells in a suitable medium to propagate the live attenuated chimeric Zika virus; and
- c) harvesting the live attenuated chimeric Zika virus.

Additional steps may of course be added, either antecedent, intermediate or subsequent steps. The method for example advantageously comprises a subsequent step of purification of the harvested virus. A step of pharmaceutical formulation may also be added, at the end of the method.

Different mammalian cells are well known to the skilled person for the production of viruses, especially flaviviruses. Particularly preferred mammalian cells according to the invention are VERO cells, for example Vero cells ATCC CCL-81. Alternative suitable cells are BHK-21 (for example ATCC CCL-10), C7/10 cells, LLC-MK2, FRhL or MRC-5 cells, or any appropriate mammalian cells, or cells of mosquito origin.

A suitable medium is *inter alia* a serum free medium. The components of the medium are to be adapted depending on the cells to be used in the method.

According to a preferred embodiment, step a) of the method is carried out by transfecting the cells with the RNA obtained by transcription of a vector, especially a plasmid, as characterized above.

The recovery of the virus at step c) is preferably in the culture supernatant.

The live attenuated chimeric Zika virus, or the recombinant sequence allowing the production of such a virus, is as defined in the other aspects of the invention. The preferred features of such a virus have been detailed in the preceding sections and these features are all applicable to the present aspect of the invention. It is moreover particularly preferred that the chimeric virus, or the DNA sequences coding for its viral genome, comprises a sequence encoding a hybrid yellow fever–Zika C-prM signal sequence having the sequence of SEQ ID NO: 6, or a sequence having one or two mutations with respect to this sequence. The different advantages of these sequences have been detailed in the previous sections. The inventors have moreover determined that the hybrid sequence generally achieves better growth performance of the virus, especially in Vero cells.

It is understood that the various features and preferred embodiments of the present invention as disclosed herein may be combined together.

The present invention will be further illustrated by the following examples. It should be understood however that the invention is defined by the claims, and that these examples are given only by way of illustration of the invention and do not constitute in any way a limitation thereof.

EXPERIMENTAL SECTION:

The examples below provide evidence of viability and efficient replication *in vitro*, i.e. manufacturability of various Chimeric Zika (CYZ) designs. Based on the obtained data, these YF 17D-based chimeras have moreover the potential to be highly attenuated while remaining highly immunogenic and efficacious in animal models and humans.

Evidence of high attenuation without loss of a sufficient immunogenicity in mice is provided herein; i.e. the proof that the right balance between attenuation and immunogenicity is compatible with ChimeriVax Zika design.

Evidence from standard models developed previously for other ChimeriVax vaccines is believed to be indicative of safety and effectiveness of a live Zika vaccine. The present results also tend to show that there would be no sexual transmission of the Chimerivax Zika construct.

The present inventors have also optimized the C-prM signal sequence, demonstrating that a specific hybrid yellow fever–Zika C-prM signal sequence improves the immunogenicity of the virus, while preserving the attenuation of the virus and its growth performance.

Example 1. Materials and methods:

- Construction of CV-Zika variants

YF/Zika chimeras containing the French Polynesian (FP) (GenBank accession number KJ776791) prM-E genes in the YF17D backbone were generated by replacing the TBE-Hypr specific prM-E-genes with the corresponding FP genes in ChimeriVax-TBE (p41 plasmid). The FP genes for constructs p391 and p392 were synthesized by DNA2.0, Inc. (Menlo Park, CA) and cloned using restriction sites SphI –MluI in the ChimeriVax-TBE plasmid. Modifications to the nucleotide sequence to introduce M60 mutation (p401), glycosylation site removed in envelope (p429), C2 deletion in YF capsid gene (p428), and deletion in the YF 3'UTR (p398) were constructed with synthesized gene blocks by Integrated DNA Technologies (IDT, Iowa). Variants p391, p392, p401, and p398 were engineered with the YF prM signal sequence, whereas p393, p394, p429, and p428 contain a hybrid YF/FP prM signal sequence based on cleavage prediction scores when sequences were analyzed by the software program SignalP version 3.0. Constructs containing the hybrid prM signal sequence were generated with synthesized gene blocks (IDT). Resulting plasmids were linearized with XhoI and *in vitro* transcribed using Amplicap SP6 High Yield message Maker kits (Cellscript). *In vitro* transcribed RNA was then transfected into Vero cells to generate infectious virus.

- Removal of the glycosylation site by amino acid substitution

The 3D coordinates of the Zika virus envelope protein were obtained from the Protein Data Bank and used as the basis of mutant design (PDB ID: 5IRE Sirohi et al. Science 352:467-470; www.rcsb.org H.M. Berman et al (2000) The Protein Data Bank Nucleic Acids Research, 28: 235-242.).

This PDB structure was prepared for modeling by building missing parts, assigning hydrogens, fixing structural defects and refining the structure. Suitable mutations intended to remove the glycosylation site at position N154 were identified using BioLuminate (Release 2015-3, Schrödinger, LLC, New York, NY, 2015). The *in silico* mutation process involved scanning the protein for potential residue mutations, generating 3D structures of the mutant forms, and comparing the properties of the mutated structures. In each case, the protein structure around the mutation site was allowed to relax in response to the amino acid substitution. Relaxation was achieved by minimizing the energy of the newly introduced residue and those around it, with solvent effects accounted for using an implicit solvation model. Surrounding residues selected for inclusion in the refinement step were defined as those within a cutoff distance of 4 Angstroms of a hypothetical Arg at the mutation site. The use of a hypothetical Arg ensured that the same set of residues was refined regardless of the identity of the initial or mutated residue and thus, calculated properties of mutants could be compared fairly.

Change in the stability of the protein as a result of mutation was computed using the Prime energy function with an implicit solvent term. For the purpose of selecting the best mutants, stability was defined as the difference in free energy between the folded state and the unfolded state. All mutants predicted to be stable were further analyzed by visual inspection in order to make the final selection that was forwarded for experimental validation.

- Virus recovery and *in vitro* characterization

CV-Zika viruses were recovered and propagated in normal Vero cells in serum free VPSFM media (Gibco) and recovered virus stocks were supplemented with sorbitol to a final concentration of 10% and stored at -80°C. Growth curve analysis of CV-Zika variants was done in normal Vero cells in serum free conditions where cells were infected at an MOI 0.1, 0.01, and 0.001 for 1 hour at 37°C, 5.0% CO₂ in VPSFM media, and overlaid after virus absorption with VPSFM supplemented with antibiotic-antimycotic. Titers (pfu/ml) were determined on growth curve samples collected between day 1 and day 7.

Titers of viruses were determined on Vero cells either using standard titration with crystal violet (Guirakhoo et al, 1999 and Monath et al, 2006) or by immunostaining with an anti-Zika envelope Mab. Briefly, cells in 24-well plates were infected by serially diluted viruses for 1 hr, and overlaid with Vero cell growth medium with 2% FBS supplemented with 0.84% methylcellulose (Invitrogen). On day 5 post-infection, cells were fixed with methanol for 1 hour at room temperature and virus foci/plaques were probed with Zika Envelope MAb0502156 (Aalto Bioreagents Ltd) and detected with an anti-mouse IgG-HRP (Thermo Fisher Scientific) conjugated secondary antibody. Serial passages of CV-Zika variants to amplify and assess genetic stability of the insert were done in Vero cells at a controlled MOI 0.01-0.001 infection. Sequence analysis of cDNA generated from passaged CV-Zika viruses was done to verify nucleotide sequence.

- Mouse Studies.

All procedures were performed under approved IACUC protocols in accordance with the National Institutes of Health requirements for humane treatment of laboratory animals. ICR mice were from Charles River (Charles River Laboratories International, Inc., Wilmington MA, USA) Inoculation routes/doses, and bleeding days were as described in examples 3-5.

- Mouse Neurovirulence Studies.

Five-day-old suckling mice, in groups of 9 to 12, or the three to four-week-old mice in groups of 8, were inoculated by the intracranial (i.c.) route with wt ZIKV, CYZ, LAV controls or mock controls at the indicated doses in 20 µl of MEM containing 0.25% human serum albumin using 0.5-ml Hamilton Syringe with Luer Tip, 27G 1/4" stainless steel reusable microneedles. Inoculated mice were observed for symptoms of encephalitis, including ruffled hair, hunched back, paralysis, and death. Paralyzed, moribund mice were euthanized and scored during the three-week observation period. Average survival times were calculated for animals that succumbed to infection. Virus doses inducing 50% mortality were calculated using the Reed and Muench method.

- Immunogenicity in Mice.

3.5-week-old ICR mice were immunized by the IP route with the indicated test articles and controls (8 animals per group). Mice were boosted on day 28 with the same test articles by the same route used for prime immunization. Animals were bled on days 27 and 53 and heat-inactivated serum samples were tested in a standard PRNT₅₀ assay against wt ZIKV strain Puerto Rico-2015 or strain Uganda MR766 to determine neutralizing antibody titers.

- Plaque reduction neutralization test (PRNT).

Approximately 100 PFU of ZIKV were mixed 1:1 with mouse serum serially diluted in a final volume of 240 µl of MEM. The mixture was incubated at 37°C for 1 h prior to use for infection of Vero cells in duplicate wells of a 24-well plate (100 µl/well). Infected Vero cells were added with a medium overlay containing 1% FBS, 2mM L-glutamine (Gibco), 1% methyl-cellulose (Fluka), and 1x Anti-Anti (Gibco) and incubated at 37°C for 4 days. Cells were then fixed with methanol and virus plaques were visualized by gentian violet/methanol staining. Calculations of end point titers were performed using GraphPad Prism 6 (GraphPad Prism Software, Inc., San Diego, CA).

- Replication of Zika viruses in human neuroblastoma cells

Human SK-N-SH neuroblastoma cells were obtained from ATCC and maintained in Eagle MEM medium (Invitrogen) supplemented with 10% FBS.

The kinetics and level of replication of the wt ZIKV strains and the CYZ variants were compared in SK-N-SH cell lines. Cells grown on T-25 flasks were infected with virus at an MOI of 0.01 or 0.001 PFU per cell and were allowed to adsorb for 1 h at room temperature, after which fresh medium was added. Infected cells were incubated at 37°C. Virus in culture medium was harvested daily, and the titer was determined in Vero cells. Infectious virus titers were determined after 4 days of incubation using the plaque assay.

- Titration of Zika-neutralizing antibodies:

Ten week-old male A129 mice provided from B&K Universal were injected by subcutaneous route in the scapular belt under 100µL. Blood samples were performed in presence of EDTA and plasmas were collected 26 days post-infection for humoral responses.

ZIKA-neutralizing antibodies in the sera were titrated by microneutralization assay by mixing dilutions of heat-inactivated sera (56°C for 30 min) with an equal volume of medium containing ZIKA (25 CCID50/well) and then incubating for 75 min at 37°C. An aliquot of serum/virus mixture (100 µL) was then added to Vero cell monolayers in flat bottom 96-well plates and incubated in a 37°C, 5% CO₂ cell culture incubator for 4 days. Infected cells were fixed with acetone and immuno-stained with biotinylated 4G2 monoclonal, streptavidine conjugated to alkaline phosphatase, and developed with BCIP/NBT (Sigma-Aldrich) chromogenic reagents. Titers were calculated from the dilution that resulted in 50% neutralization (SN50) by using a least-square regression method.

- ELISA antibody responses.

96-well microplates were coated overnight at 4°C with 100 ng/well (100 µL) of inactivated WT Zika in phosphate buffered saline (PBS) buffer. Plates were then blocked for 1 hour at 37°C with 150 µL of PBS-Tween-milk. All further incubations were carried out in a final volume of 100 µL, followed by 4 washings with PBS-Tween. Serum samples that were serially diluted twofold in PBS-Tween-milk beginning at 1/100 or 1/1000 were added to the wells and incubated for 90 min at 37°C. After washing, HRP-conjugated antibodies to mouse IgG were added and the plates were incubated for another 90 min at 37°C followed by washing and color development with TMB substrate. The optical density (OD) was measured at 450-650 nm with an automatic plate reader. Antibody titers were defined by calculating the reciprocal dilution which gives an OD of 1.0.

Example 2. Designs, construction and *in vitro* replication of ChimeriVax-Zika (CYZ) candidates.

Prior to CYZ constructions, phylogenetic analysis was done by the inventors on available wild type (wt) Zika virus sequences. French Polynesia-2013 strain (FP2013), corresponding to the sequence having accession number KJ776791.1, was chosen as a donor strain because the amino acid sequence of its prM-E genes was identical to most recent Brazilian (2015), Haiti

(2014), Martinique (2015) and Puerto Rico (2015) strains, and almost identical to many others. The amino acid sequence of the M and E proteins of the FP2013 strain has moreover been identified by the inventors as corresponding to the Asian/American consensus sequences (as generated by the inventors via alignment of multiple sequences from different Zika viruses), with respect to the M and E proteins.

The prM-E genes of FP2013 strain were synthesized and used to replace the TBE specific prM-E genes in a previously constructed plasmid p41 for ChimeriVax-TBE candidate (Rumyantsev et al., 2013), which is based on a low-copy number pBeloBac11 plasmid vector and is a derivative of pBSA-AR3, a single-plasmid infectious clone for ChimeriVax-JE (Rumyantsev et al., 2010). This resulted in initial p391 variant of CYZ (Fig. 1, top panel).

The plasmid was transcribed with SP6 RNA polymerase and the *in vitro* RNA transcripts were used to transfect Vero cells using Lipofectamine reagent followed by overlaying the cells with a serum free medium.

In the following p391 is indifferently used to refer to the plasmid p391 and to refer to the corresponding infectious virus, for example produced by cells transfected by the RNA transcripts corresponding to the plasmid p391.

Infectious p391 virus was detected in the supernatant of transfected cells (passage P0) and subsequent passages. The virus formed plaques in Vero cells, and infected cells were efficiently stained with anti-Zika E antibodies confirming antigenic identity (Fig. 2a and 2b). Immunostaining of cells infected with this CYZ variant, and other CYZ variants was done using a panel of Zika-E protein specific MAbs (from Aalto Bio Reagents, Ltd; for instance Mab 0502156 used at dilution 1:8,000) or a Zika-specific HIAF (from R. Tesh, WRCEVA, UTMB, Galveston, TX).

These results confirm that the chimeric viruses illustrated in Fig.1, are chimeric Zika virus in view of their antigenic identity.

In a growth curve experiment, p391 virus replicated to high titers in excess of 7 log₁₀ PFU/ml in Vero cells in serum-free medium at MOIs ranging from 0.001 to 0.1 PFU/cell.

This result indicates that the p391 virus has good manufacturability (Fig. 2c).

The prM-E genes were sequenced at P2 passage level and the sequence was found to be as expected. This result indicates that p391 also has good stability.

It is to be noted in this respect that the design contains a silent A to T nucleotide change at nucleotide 166 (Gly codon of the YF 17D C protein amino acid 15) which originated in the starting p41 plasmid. This nucleotide change is located close to the RNA cyclization sequence but is considered acceptable as it is also present in several published wt YF sequences. To additionally confirm that this mutation is inconsequential to CYZ replication, the change was reverted (in variant p411) and the recovered virus had the same replication characteristics in Vero cells as p391 and appeared to replicate equally efficiently *in vivo* (in mice; see below).

Additional viable chimeras were similarly constructed, namely p395 and p396, containing the prM-E genes from representative Asian (Cambodia-2010, GenBank accession number JN860885) and African (Senegal-1984, GenBank accession number HQ234501) ZIKV strains, respectively, to serve as controls. These viruses replicated as efficiently as p391 FP2013 chimera in Vero cells.

These variants were isolated prior to the recent outbreaks of GBS and congenital disease in the Pacific and Americas, and are thus supposed to be less virulent than most recent strains, such as FP 2013, or the very recent Brazil 2015 strains.

In the p391 variant, the signal sequence for prM protein (SS), i.e. the signal sequence residing between the sequence coding for the capsid protein and the sequence coding for the prM protein in a flavivirus genome, this signal sequence is YF 17D-specific. Computer predictions with SignalP program indicated that swapping this SS with a hybrid YF/Zika SS could increase the rate of the C/prM signalase cleavage of the CYZ virus polyprotein. The inventors thus hypothesized that this could increase virus replication, and therefore constructed p393 variant with hybrid SS (Fig. 1).

The sequence of the hybrid sequence signal is the following:

SHDVLTVQFLILGMLLTAMA (SEQ ID No: 6), wherein the 16 first amino acids are from Yellow Fever virus and the last four (TAMA) are those of the ZIKV signal sequence, which are different from the last four amino acids naturally present in YFV, namely MTGG.

The virus was efficiently recovered in Vero cells (Fig. 3a and 3b). Importantly, it replicated to very high peak titers, in excess of 8 log₁₀ PFU/ml in Vero cells (T25 flask), e.g. on day 5 at MOI 0.001 (Fig. 3c). The P2 passage of p393 virus was sequenced and shown to have no mutations in prM-E genes.

p392 and p394 derivatives of p391 and p393 variants were constructed (Fig. 1), respectively, containing three amino acid changes corresponding to the three attenuating SA14-14-2 JE vaccine-specific amino acid changes in the E protein that had been used in ChimeriVax-WN vaccine to increase attenuation (E-107 L to F, E-316 A to V and E-440 K to R in ChimeriVax-WN02 human vaccine) (Arroyo et al. 2004; Monath et al. 2006) (corresponding to positions 107, 319 and 443 of p391/393 and p392/394).

An additional variant was constructed, p401, which is the same as p392 but also containing a mutation in the ZIKV M protein corresponding the M-60 K to C adaptation in ChimeriVax-JE (IMOJEV™) which genetically stabilized the vaccine during manufacturing in Vero cells and increased the yields by ~10 fold for ChimeriVax-JE (Fig. 1).

The three variants were successfully recovered in Vero cells, and their genetic stability was evaluated by serial passages in Vero cells. During passaging, one of the three WN02 residues in p392 chimera, E-107, reverted to wild type Leu (after two passages, both sequences F and L

were found and after 4 passages, all viruses reverted to L), while the E-316 (corresponding to E-319 in CYZ) and E-440 (corresponding to E-443 in CYZ) changes remained.

The resulting p392 double-mutant variant is thus more stable than the triple-mutant.

It may also be possible to stabilize the E-107 L to F change by plaque-purification of virus; a stabilizing effect of this step has been described previously (Pugachev et al. 2004).

To evaluate whether attenuation of CYZ can be enhanced by de-glycosylation of the E protein, a mutation ablating the N-linked glycosylation motif (Asn-X-Ser/Thr motif) located at E-154 position was designed based on the lowest energy prediction. By *in silico* mutagenesis, the following results, detailed in table 1, have been obtained regarding the variation of stability (Δ stability).

Table 1: variation of stability for different substitutions of N154 in E protein.

mutation	N154	N154A	N154D	N154E	N154Q
Δ stability	NA	-0.1	7.8	-5.3	-7.8

Based on energy calculation (the negative variations of stability are favored) and visual inspection, the N154Q mutation was chosen by the inventors. Alternative options, on the basis of these criteria are N154A and N154E.

The mutation N154Q was combined with the hybrid YF/ZIKV signal for prM (as in p393) resulting in construct p429 (Fig. 1). Viable virus was recovered following transfection and shown to form plaques and grow to titers above 7 log₁₀ PFU/ml in serum free Vero culture (Fig. 4). The mutation was confirmed by virus sequencing.

The glycosylation site in CYZ can also be ablated using natural modifications that have been observed in wt Zika viruses during their adaptation to substrates, e.g. specific amino acid deletions or point mutations that occurred spontaneously during propagation in mouse brain tissue (Lanciotti et al. 2008; Faye et al. 2014).

Finally, the inventors have previously identified various modifications of the YF 17D backbone, specifically small deletions in the C protein and deletions in 3'UTR, that can be used to achieve additional, minor attenuating effects in already attenuated chimeric viruses as shown using ChimeriVax-WN candidates (WN04 mutations, WO2006/116,182). One such promising WN04 mutation, a 3-amino acid deletion C2 in the YF 17D C protein (deletion of PSR residues at amino acids 40-42) was introduced in CYZ. The deletion was combined with hybrid SS for prM (as in p393) resulting in variant p428 (Fig. 1). The recovered virus formed plaques and grew to more than 7 log₁₀ PFU/ml in serum-free Vero cells (Fig. 5b). The deletion was confirmed by virus sequencing.

Another promising WN04 attenuating mutation, a 5-nt deletion dB in the 3'UTR (nucleotides 256-260 from the 3'end, CAGGT, deleted to destabilize a predicted secondary RNA structure

element) was introduced resulting in variant p398 (Fig. 1). p398 virus was shown to be viable right after transfection (6 log₁₀ PFU/ml titer at P0). Other WN04-based designs can include double-mutants of CYZ, for example, a CYZ variant with a previously identified promising combination of the C2 capsid protein deletion and a 3'UTR deletion d7 (nucleotides 345-351 from 3'end, AAGACGG; variant p399), or other combinations.

Example 3. CYZ attenuation in mouse neurovirulence (NV) tests.

The YF 17D virus, a licensed human vaccine, is the standard comparator of safety for new flavivirus vaccine candidates whose virulence in animal models should not exceed that of YF 17D. YF 17D is neurovirulent for mice of all ages, while previously constructed ChimeriVax vaccines against JE, DEN and WN are significantly more attenuated. ChimeriVax vaccines are generally not neurovirulent for adult mice, while the more sensitive suckling mice are susceptible in a dose- and age-dependent fashion, at doses several orders of magnitude higher than YF 17D (Pugachev et al. 2010; Guy et al., 2010; Guirakhoo et al., 1999; Arroyo et al., 2004).

It should be noted that most ZIKV isolates are not neurovirulent in mice unless they are mouse-brain adapted by serial brain-to-brain passages (Dick. 1952). It is thus of outmost importance to prove that no neurovirulence is imported into the chimeric Zika constructs by the Yellow Fever backbone.

Attenuation of CYZ-FP2013 variants p391, p393 and p411 was compared in a highly sensitive suckling mouse NV test to the YF 17D benchmark (YF-VAX derived preparation), as well as wt ZIKV strain Puerto Rico-2015 (having an identical M and E proteins sequences and differing at only 7 nucleotide positions from the FP2013 strain) and a highly attenuated ChimeriVax-JE control (research preparation). MEM was also used as control.

Five-day old ICR suckling mice were inoculated with graded doses of viruses or control by the intra-cerebral (IC) route (1 dam per group; suckling mice randomized between dams). The mice were observed and mortality was recorded after 21 days.

The intermediate results for survival at day 12 are reported in table 2 and the final results are illustrated in Fig.6. Figure 13 compares the numbers of sick and healthy animals in the groups receiving the p393 and the wild type ZIKV PR viruses (the main signs of illness considered were hunched posture, ruffled fur, weight loss and neurological manifestations). As can be seen from Figure 13, the p393 virus is more attenuated than the wild type ZIKV PR virus.

Table 2: intermediate results at day 12, for different tested viruses

Virus	Dose (Log ₁₀ PFU)	Survival (%)	Additional observation
p391	4	10/10 (100)	

Virus	Dose (Log10 PFU)	Survival (%)	Additional observation
p391	3	10/10 (100)	
p391	2	11/11 (100)	
p391	1	11/11 (100)	
p391	0	11/11 (100)	
p391	-1	11/11 (100)	
p393	4	10/10 (100)	
p393	3	10/10 (100)	
p393	2	11/11 (100)	
p393	1	12/12 (100)	
p393	0	11/11 (100)	
p393	-1	11/11 (100)	
p411	4	11/11 (100)	
p411	3	11/11 (100)	
p411	2	10/10 (100)	
p411	1	10/10 (100)	
p411	0	10/10 (100)	
p411	-1	10/10 (100)	
WT ZIKV Puerto Rico	4	11/11 (100)	6 mice sick (54%)
WT ZIKV Puerto Rico	3	11/11 (100)	1 mouse sick (9%)
WT ZIKV Puerto Rico	2	10/10 (100)	1 mouse sick (10%)
WT ZIKV Puerto Rico	1	11/11 (100)	
WT ZIKV Puerto Rico	0	11/11 (100)	1 mouse sick (9%)
WT ZIKV Puerto Rico	-1	11/11 (100)	
YF-VAX	2	0/10 (0)	
YF-VAX	1	8/11 (73)	
YF-VAX	0	9/11 (82)	
YF-VAX	-1	11/11 (100)	
ChimeriVax-JE	3	10/11 (91)	1 mouse sick
ChimeriVax-JE	2	11/11 (100)	
ChimeriVax-JE	1	11/11 (100)	
Mock (MEM)	-	11/11 (100)	

As can be seen, CYZ variants (p391, p393 and p411) caused only a few occasional deaths at some of the doses tested (the intended doses were from -1 to 4 log₁₀ PFU), which was similar to ChimeriVax-JE, as well as ZIKV strain Puerto Rico-2015 (a mouse un-adapted strain). In contrast, YF 17D caused mortality in dose-dependent fashion, as expected, with an IC LD₅₀ of 0.44 log₁₀ PFU (Fig. 6). It can be deduced that the median lethal dose LD₅₀ is greater than 4

Log₁₀ PFU for ChimeriVax Zika or wt Zika PR and that the LD₅₀ is much lower, namely 0.44 Log₁₀ PFU for YF-VAX®.

Thus CYZ viruses (LD₅₀ > 4 log₁₀ PFU) are thus more than 3,500 times less neurovirulent compared to YF 17D, and are as highly attenuated as ChimeriVax-JE.

These results demonstrate that the un-adapted wild type Zika viruses are not neurovirulent, and that the tested chimeric Zika virus tested are also not neurovirulent. The YF 17D backbone of the chimeric viruses therefore does not trigger neurovirulence of the chimeric Zika viruses.

The results obtained with p393 also show that the hybrid yellow fever–Zika C-prM signal sequence has no detrimental effect on the attenuation of the chimeric Zika virus.

Similar results were observed in a preliminary NV experiment in adult (3.5 week old) ICR mice in that CYZ p391 and p393 variants were highly attenuated compared to YF 17D (YF-VAX) as well as wt Zika MR766 (a highly mouse brain-adapted Uganda-1947 reference strain).

Specifically, CYZ p391, p393, p395 and p396 viruses, as well as different wild type Zika strains, including the highly mouse brain-adapted Uganda were inoculated intra-cerebrally, at different doses, and mortality was recorded after 28 days. CYZ p391 & p393 were avirulent at a 4 log₁₀ PFU dose, while MR766 killed all mice at the same dose in 6-10 days, and YF 17D, which was tested at graded doses, caused mortality with an IC LD₅₀ of ~ 1 log₁₀ PFU. Thus neurovirulence of the CYZ variants is more than 1000 times lower compared to the YF 17D benchmark in this test. Furthermore, it should be noted that the p391 and p393 constructs (based on the FP2013 Zika strain sequence) were unexpectedly even more attenuated than the p395 and p396 constructs (which were based on Zika strains isolated in Cambodia and Senegal respectively). For example, when p391, p393, p395 and p396 constructs were administered at doses of 2.8, 2.4, 1.7 and 1.4 log₁₀ PFU respectively, higher mortality rates were observed for the p395 and p396 constructs. The full results at day 28 of the experiment are reported in table 3 below.

Table 3:

Virus	Dose (Log10 PFU)	Survival (%)	% mortality
p391	4.14	8/8 (100)	0
p391	2.8	7/8 (87.5)	12.5
p393	4.5	8/8 (100)	0
p393	2.4	7/8 (87.5)	12.5
p395 (Cambodia)	4	7/8 (87.5)	12.5
p395 (Cambodia)	1.7	4/7 (57.1)	42.9
p396 (Senegal)	3.3	6/8 (75)	25
p396 (Senegal)	1.4	5/8 (62.5)	37.5
WT ZIKV Mexico 1-44	3.3	7/8 (87.5)	12.5

Virus	Dose (Log10 PFU)	Survival (%)	% mortality
WT ZIKV Cambodia FSS 13025-44	3.5	8/8 (100)	0
WT ZIKV Malaysia P 6-740	2.6	8/8 (100)	0
WT ZIKV Senegal DAKAR 41519	2.6	4/8 (50)	50
WT ZIKV Uganda (MR766)	3.4	0/8	100
YF-VAX	1.6	8/8 (100)	0
YF-VAX	0.9	5/8 (62.5)	37.5
YF-VAX	0	7/8 (87.5)	12.5
YF-VAX	-1	8/8 (100)	0
CV-JE	4.6	8/8 (100)	0
Mock (PBS)	0	8/8 (100)	0

Example 4. CYZ attenuation, immunogenicity and protective efficacy in IFN receptor deficient mice (A129 and AG129) following peripheral inoculation (Experiments 1-3).

Experiment 1: ZIKV has been shown to replicate in various organs and cause disease in IFN receptor-deficient mice (i.e. immunodeficient mouse strains A129, AG129, *Ifnar*) (Rossi SL et al, 2016; Aliota MT, et al, 2016; Lazear HM, et al, 2016), thus these mouse strains could be developed as models to compare attenuation of CYZ candidates to wt ZIKV.

Immune responses to ZIKV in immunodeficient mice have not been reported. However IFN-deficient mouse strains have been successfully used in studies on Dengue pathology and vaccines (Zompi S, et al, 2012). Therefore they were used to evaluate immunogenicity and efficacy of CYZ and other Zika strains.

The inventors inoculated A129 mice lacking type I interferon receptor (IFN α and β) and AG129 mice deficient in both type I and II interferons (IFN α , β and γ) with 5 log₁₀ PFU of CYZ variant p393 by the SC (subcutaneous) route. All AG129 mice became sick, lost weight, and died or were euthanized by day 13. In contrast A129 mice did not die and remained healthy. This is similar to YF 17D virus that has been shown to be lethal for AG129 mice but not A129 mice (Meier KC, et al, 2009), and in contrast to wt ZIKV strain Cambodia-2010 that has been shown to kill both AG129 and A129 mice in age /dose-dependent fashion (Rossi et al, 2016).

Thus this experiment with CYZ (p393) indicated that CYZ has an attenuated virulence profile which is similar to YF 17D, and that it is more attenuated than wt ZIKV in this model.

It should be noted that wt ZIKV (Puerto Rico-2015) control was also included in the experiment but administered at a low dose (< 2 log₁₀ PFU based on back-titration of inoculate). The virus did, however, replicate rather efficiently as evidenced by clinical signs and mortality in mice and the presence of virus detectable by Zika NS5-based RT-qPCR in most organs where CYZ was also

detected on day 5 (liver, spleen, brain, testes). Zika NS5 qRT-PCR was performed using the following primers and probe:

Name	Type	Sequence
ZIK-P2	Probe FAM-MGB/NFQ	YCAYCACTTCAACA (SEQ ID NO: 11)
ZIK-F2	Forward Primer	GGAAGARGTYCCGTTYTG (SEQ ID NO: 12)
ZIK-R2	Reverse Primer	GCCAATCAGTTCATCTTG (SEQ ID NO: 13).

One important observation was that in AG129 mice that were euthanized on days 12-20 (because of severe disease), viral loads were similar for both CYZ and wt ZIKV in spleen, liver (very low level) and brain tissues, while CYZ loads in spleen and brain of A129 mice was reduced compared to wt ZIKV on day 26 (end of study) (Fig. 7, panels A-C). The latter is indicative of CYZ attenuation in A129 mice. Most strikingly, very high loads of wt ZIKV were detected in testes of both AG129 and A129 mice, while titers of CYZ were very low or undetectable (Fig. 7, panel D). The latter is relevant to recent reports of sexual transmission of ZIKV and persistence of the virus in semen of men up to 6 months after infection.

The data in immunodeficient mice indicates that in contrast to wild type ZIKV, the chimeric Zika virus does not replicate efficiently and does not persist in testes, which is an important safety feature.

Moreover, the endpoint viremia was lower in those mice that had received CYZ when compared to the viremia levels in those mice that had received wild type ZIKV (data not shown), despite the fact that the dose of CYZ that was administered was higher. These data provide further evidence of the attenuation of CYZ.

A129 mice immunized either with CYZ (namely p393) or wt ZIKV (Puerto-Rico 2015) developed high-titer, and rather uniform Zika specific antibody responses, confirming that the chimeras are as immunogenic as the wild type Zika virus.

The antibody responses were measured by ELISA and SN₅₀ (micro-neutralization titration of serum neutralizing antibodies; SN₅₀ titers generally correlate with PRNT₅₀ titers) on day 26 (Table 4). In the CYZ group, neutralizing antibody titers were in the range 100 – 252 (GMT 154 ± 156), which was similar to wt ZIKV inoculated mice (138 – 631, GMT 288 ± 287) indicative of high neutralizing immunogenicity of CYZ that is expected to provide efficient protection from ZIKV challenge.

For comparison, ChimeriVax-WN02 vaccine given to ICR mice at 5 log₁₀ PFU dose by SC route (the route used in this experiment) elicited Neutralizing Antibodies with GMT 37 ± 45 and the mice were 100% protected from severe WN virus challenge (~ 250 LD₅₀) (Arroyo et al., 2004).

Table 4:: Zika specific antibody responses in A129 mice on day 26 post-immunization.

Test article	Dose	Animal	ELISA Titer at D26 (post-1 IM)	Neutralizing Titer	
			anti-ZIKV IgG Titer (UE)	(Log ₁₀ SN50)	(SN50)
CYZ	10 ⁵ CCID50	28	3,937	2,3	200
		29	4,204	2,3	200
		30	4,257	2,4	252
		31	3,863	2,0	100
		32	3,976	2,1	126
		33	4,097	2,2	159
		34	4,184	2,0	100
		Geometric mean	4,074	2,2	154
		Standard deviation	0,151	2,2	156
WT ZIKV PR	<10 ^{1.6} CCID50	38	5,310	2,8	631
		39	4,616	2,14	138
		40	4,689	2,26	182
		41	4,689	2,8	631
		42	4,685	2,43	269
		43	4,564	2,36	229
		44	4,677	2,43	269
		Geometric mean	4,747	2,5	288
		Standard deviation	0,253	2,4	287
Saline 0.9 % + Sorbitol 10%	N/A	1	1.000		
		2	1.000		
		23	1.000		
		24	1.000		
		Geometric mean	1,000		
		Standard deviation	0,000		

Experiment 2. Attenuation of CYZ following peripheral inoculation was also evaluated in more susceptible, younger A129 mice. Specifically, 3-4-week old A129 mice received 5 log₁₀ PFU of CYZ variant 393 by the SC route. Control mice received the same dose of wt ZIKV Puerto-Rico-2015 or YF 17D; weight loss/mortality were expected in these control groups based on previous observations. As shown in Fig. 9, wt ZIKV Puerto-Rico-2015 virus caused a significant reduction in body weight (Panel A), neurological signs, and 87.5% mortality (Panel B). In contrast, CYZ p393 virus was found to be highly attenuated as no weight loss, sickness or deaths were observed [p<0.05; Log-rank (Mantel Cox) test]. YF17D was also attenuated in this experiment as only 1 out of 8 mice died.

Experiment 3. Biodistribution, immunogenicity as well as protective efficacy of CYZ were examined in an additional experiment in A129 mice. Male and female 8-week old A129 mice were inoculated with CYZ 393, 392, 428 and 429 variants, or wt ZIKV Puerto-Rico-2015 control by the SC route at a dose of 5 log₁₀ PFU for each virus. Biodistribution of CYZ 393 and ZIKV Puerto-Rico-2015 viruses was compared on days 5 and 26 by measuring viral RNA loads in the spleen, liver, brain and testicles (of males) using RT-qPCR in 3 animals per group/time point. High viral RNA loads of ZIKV Puerto-Rico-2015 were observed on day 5 in all organs with RNA concentrations of ~ 4.5 – 6.5 GE/mg of tissue, while the CYZ 393 vaccine candidate was significantly attenuated as viral loads were ~ 2.5 order of magnitude lower in the spleen, liver and testicles, and importantly no CYZ virus was detected in the brain (Fig. 10, day 5). On day 26, ZIKV Puerto-Rico-2015 was detectable in all the organs, with particularly high RNA loads in testes and brain (~ 6 and 4 GE/mg, respectively), while CYZ RNA was only detectable at very low levels in the spleen (Fig. 10, day 26). This demonstrates that CYZ virus is significantly attenuated compared to wt ZIKV in this model, and that the replacement of the NS protein genes with the YF17D-specific NS genes in the chimeric vaccine resulted in the inability of CYZ to persist in peripheral organs, particularly in the liver and testes. It is possible that some of the ZIKV NS proteins can suppress the innate and/or adaptive responses in some (immuno-privileged) organs allowing wt ZIKV to persist, while YF17D specific NS proteins in the chimera disable this immunosuppressive effect resulting in rapid virus clearance and thus CYZ attenuation.

The CYZ vaccine candidates 393 and 392 elicited high neutralizing antibody responses against both Puerto Rico-2015 and MR766 ZIKV viruses, indicative of the breadth of immunity provided by CYZ against geographically/genetically distant ZIKV strains (e.g. PRNT₅₀ GMTs 1,354 and 2,995, respectively, for CYZ 393); the immunogenicity of the CYZ 393 variant was comparable to that of the Puerto Rico-2015 immunizing virus (Table 5). The attenuating genetic modifications in CYZ 428 and 429 variants resulted in a reduced immunogenicity, e.g., compared to CYZ 393, yet PRNT₅₀ titers elicited by these viruses were in the hundreds and thus they should be considered viable vaccine candidates as well.

Table 5. Zika specific antibody responses in A129 mice (Experiment 3) on day 26 post-immunization.

Immunization with	PRNT₅₀ (GMT ± 95% CI) against ZIKV PR 2015	PRNT₅₀ (GMT ± 95% CI) against ZIKV MR766
CYZ FP (p392)	358 ± 106	766 ± 520
CYZ FP-SS (p393)	2284* ± 1194	2995± 2724
CYZ (p428)	712 ± 669	850 ± 362

CYZ (p429)	353 ± 335	708 ± 447
ZIKV PR	3900 ± 2151	5490 ± 8212
Mock (MEM)	<20	<20

*GMT values of p393 significantly higher than the GMT values of p392 and p429 (one-way ANOVA, $p \leq 0.05$).

To demonstrate protection, mice immunized with CYZ 393, 392, 428 and 429 variants and mock-immunized mice were challenged on day 31 with 3 log₁₀ PFU of wt ZIKV_Puerto Rico-2015 virus given SC. Post challenge viremia was measured by RT-qPCR in sera collected on days 1, 3, 4 5, 7 and 9 after challenge. CYZ immunized animals were protected as only very low RNA levels were detectable in some animals (which was not unexpected given the very high sensitivity of RT-qPCR). This was in contrast to mock immunized controls where high post-challenge viremia was observed on days 3 – 9, peaking at ~ 7 log₁₀ GE/ml on day 7 ($p < 0.002$; Mann-Whitney test) (Fig. 11).

Example 5. CYZ immunogenicity and protective efficacy in immunocompetent mice following peripheral inoculation.

3.5-week old ICR mice were immunized with CYZ variants p391, p393, p395 (Cambodia-2010), and p396 (Senegal-1984) with 5 log₁₀ PFU given via the intra peritoneal (IP) route, either as a single dose or by 2 doses on days 0 and 28. Two doses were used on the basis of the ChimeriVax-DEN vaccination scheme. Different wild type ZIKV strains, namely Mexico I-44, Cambodia FSS 13025, Senegal DAK AR 41519 and Uganda MR766 were also used for comparative purposes, as well as PBS.

All animals remained healthy throughout the study. After one dose (day 27), CYZ immunized animals had very high Zika-specific N Ab titers determined against Puerto-Rico-2015 and Uganda-1947 (MR766) strains (GMTs ranging from 1282 to 7389). After two doses determined on day 21 after the second dose (day 49 of the study), all immunized mice with CYZ variants had PRNT₅₀ titers > 2000, irrespective of the strain used for the determination of the PRNT₅₀ titers, indicative of very high immunogenicity. The results are presented in table 6 (single dose of ChimeriVax or controls) and table 7 (two doses of ChimeriVax or controls).

Table 6: Immunogenicity of a single dose of ChimeriVax Zika in 3.5 week-old ICR mice

Immunization with:	PRNT ₅₀ (GMT ± SD / 95% CI) against ZIKV Puerto Rico	PRNT ₅₀ (GMT ± SD / 95% CI) against ZIKV MR766
CYZ FP (p391)	1868 ± 1739 / 2766	1282 ± 1165 / 1854
CYZ FP-SS (p393)	7389* ± 8630 / 13732	3774* ± 1796 / 2858
CYZ Cambodia (p395)	2998 ± 1480 / 2355	1562 ± 1040 / 1654
CYZ Senegal (p396)	1421 ± 1352 / 2152	2079 ± 1701 / 2706

ZIKV Mexico I-44	727 ± 1956	392.6 ± 531
ZIKV Cambodia FSS 13025	117 ± 55.41	¹ 329.4 ± 290
ZIKV Senegal DAK AR 41519	2558 ± 3980	2528 ± 1607
ZIKV Uganda MR766	<100	1551 ± 722
Control (MEM)	<100	<100

¹The PRNT₅₀ values were calculated based on two pools of sera

*The GMT values of p393 were significantly higher than the GMT values of p391 and p395 (one-way ANOVA, $p \leq 0.05$).

Table 7: Immunogenicity of two doses of ChimeriVax Zika in 3.5 week-old ICR mice

Immunization with:	PRNT ₅₀ against ZIKV Puerto Rico	PRNT ₅₀ against ZIKV MR766
CV ZIK FP (P391)	≥10,000	2,000
CV ZIK FP-SS (P393)	≥10,000	≥10,000
CV ZIK Cambodia (P395)	≥10,000	≥10,000
CV ZIK Senegal (P396)	≥10,000	≥10,000
Mexico I-44	≥10,000	≥10,000
Cambodia FSS 13025	≥10,000	≥10,000
Senegal DAK AR 41519	≥10,000	≥10,000
Uganda MR766	1,047	≥10,000
Control (PBS)	<20	<20

A comparison can be made to the Chimerivax-JE vaccine, which elicited neutralizing antibodies at a titer of approximately 10,000 (4 log₁₀), i.e. similar to CYZ, when administered to ICR mice at a dose of 5 log₁₀ PFU via the IP route (Rumyantsev et al., 2011). Chimerivax-JE has been shown to be highly effective in clinical trials in inducing neutralizing antibodies against Japanese encephalitis virus in humans and is now licensed for the prevention of JE infection in multiple countries.

It was recently shown that immunocompetent balb/c mice immunized with an inactivated Zika vaccine candidate (with alum adjuvant) developed neutralizing antibody titers of 15 as determined by micro-neutralization assay. Such low-level N Ab titers were sufficient to protect animals from surrogate challenge with ZIKV by the IV route (Larocca et al., 2016).

Thus there is no doubt that CYZ immunized ICR mice are protected in such a challenge model, in view of the very high neutralizing antibody titers obtained.

These very good results tend to demonstrate that the attenuated chimeric Zika virus which has been developed will be protective when used as a vaccine for mice.

The results obtained after one dose are also very interesting. All the CYZ candidates induced high levels of neutralizing antibodies, the best candidate being the p393 construct with the highest N Ab titers against both an Asian/American Zika strain (Puerto Rico) and also an

African Zika strain (Uganda MR766) after a single dose. These results are even more interesting since, as noted in Example 3, the p393 construct was observed to be more attenuated than the p395 and p396 constructs in 3.5 week old ICR mice. Normally the creation of a vaccinal strain involves a trade-off between attenuation and immunogenicity and therefore these results are unexpected.

Moreover, the observation that CYZ immunization elicited high N Ab responses against both a recent American isolate (PR-2015) and the original African isolate (Uganda-1947, i.e. MR766) indicates that the chimeric Zika vaccine will be equally effective against all Zika strains circulating in different geographical regions.

To generate experimental evidence of the efficacy of CYZ immunization, a pilot experiment was first performed in naïve ICR mice to establish a ZIKV challenge model. Approximately 6-month old ICR mice were inoculated by the intravenous (IV) route with $2 \log_{10}$ PFU of either Uganda-1947 (MR766) or Puerto Rico-2015 wild type ZIKV strains. Viremia was measured on days 1, 2, and 5 post-inoculation. Interestingly, despite the fact that Uganda-1947 (MR766) strain is highly mouse-adapted, no animals that received this virus showed viremia detectable by RT-qPCR. In contrast, four out of five mice that received Puerto Rico-2015 strain showed detectable viremia (viral RNA) on days 1 and 2 in the range of 1.08×10^3 to 2.77×10^4 genome equivalents (GE)/ml.

Therefore, the ICR mice that had been immunized with two doses of CYZ p393 variant as described above were challenged IV at ~ 7 months post-immunization with Puerto Rico-2015 ZIKV. No post-challenge viremia was detected by RT-PCR in all CYZ-immunized animals, while 3 out of 6 mice in the mock-immunized group developed viremia detected on days 1 - 3 at titers up to 5.9×10^3 GE/ml. This result demonstrates the efficacy of CYZ immunization in immunocompetent mice and also indicates that CYZ vaccination can provide immunity (as evidenced by the lack of viremia) with long duration in this model.

Example 6. CYZ replication in human neuroblastoma and neuronal progenitor (NPC) cells.

CYZ p391, p392 and p393 exhibit a 40-fold reduction in replication in human neuroblastoma cells, as can be seen from the virus titer obtained, at two different MOI, as illustrated in Fig. 8. Wild type ZIKV grew efficiently in neuroblastoma cells, reaching a titer of around $8 \log_{10}$ PFU after 3 days. When compared to these WT ZIKV, CYZ has a 40-fold reduction in titer, irrespective of the CYZ variant, compared to ZIKV.

Previous experiences with chimeric flaviviruses have indicated that a decrease in viral replication in neuroblastoma cells is generally indicative of *in vivo* attenuation, thus confirming the attenuation of the tested CYZ variants, which is also seen in human cells.

Similarly, wt ZIKV MR766 and Puerto Rico-2015 strains grew efficiently in human NPCs (MOI 0.01, titers ~ 7 log₁₀ PFU/ml or higher) which is believed to correlate with the ZIKV ability to cause pathology in immature human brain, while CYZ 391, 392 and 393 variants, as well as YF 17D control, were highly attenuated in this in vitro model as the peak titers were ~ 100 fold lower (Fig. 12).

BIBLIOGRAPHY:

Aliota MT, Caine EA, Walker EC, Larkin KE, Camacho E, Osorio JE. Characterization of Lethal Zika Virus Infection in AG129 Mice. *PLoS Negl Trop Dis*. 2016 Apr 19;10(4):e0004682;

Arroyo et al. ChimeriVax™-West Nile live-attenuated vaccine: Preclinical evaluation of safety, immunogenicity and efficacy. *J. Virol*. 2004; 78: 12497–12507

Dick. Zika virus. II. Pathogenicity and physical properties. *Trans R Soc Trop Med Hyg*. 1952 Sep;46(5):521-34;

Fauci AS, Morens DM. Zika Virus in the Americas--Yet Another Arbovirus Threat. *N Engl J Med*. 2016 Feb 18;374(7):601-4; Eurosurveillance editorial team. Resources and latest news about Zika virus disease available from ECDC. *Euro Surveill*. 2016; 21(5):32; <http://www.cdc.gov/zika/resources/index.html>

Faye et al. Molecular evolution of Zika virus during its emergence in the 20(th) century. *PLoS Negl Trop Dis*. 2014 Jan 9;8(1):e2636;

Galler et al, 1998, *Vaccines*, 16(9/10): 1024-1028;

Gubler D, Kuno G, Markoff L. Flaviviruses. In: Knipe DM, Howley PM, Griffin DE, Martin MA, Lamb RA, Roizman B, Straus SE, editors. *Fields Virology*, 5th ed. Philadelphia: Wolters Kluwer, Lippincott Williams and Wilkins, 2007. p. 1153-252;

Guirakhoo et al., Immunogenicity, genetic stability, and protective efficacy of a recombinant, chimeric yellow fever-Japanese encephalitis virus (ChimeriVax-JE) as a live, attenuated vaccine candidate against Japanese encephalitis. *Virology* 1999; 257:363-72;

Guy B, Guirakhoo F, Barban V, Higgs S, Monath TP, Lang J. Preclinical and clinical development of YFV 17D-based chimeric vaccines against dengue, West Nile and Japanese encephalitis viruses. *Vaccine* 2010; 28:632-49;

Katoh K, et al. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Res*. 2002 Jul 15;30(14):3059-66

Lanciotti et al. Genetic and serologic properties of Zika virus associated with an epidemic, Yap State, Micronesia, 2007. *Emerg Infect Dis*. 2008 Aug;14(8):1232-9;

Larkin MA, et al. Clustal W and Clustal X version 2.0. *Bioinformatics*. 2007 Nov 1;23(21):2947-8;

Larocca et al. Vaccine protection against Zika virus from Brazil. *Nature*. 2016 Jun 28. doi: 10.1038/nature18952 ;

- Lazear** HM, Govero J, Smith AM, Platt DJ, Fernandez E, Miner JJ, Diamond MS. A Mouse Model of Zika Virus Pathogenesis. *Cell Host Microbe*. 2016 May 11;19(5):720-30;
- Lindenbach** BD, Thiel H-J, Rice CM. Flaviviridae: the viruses and their replication. In: Knipe DM, Howley PM, Griffin DE, Martin MA, Lamb RA, Roizman B, Straus SE, editors. *Fields Virology*, 5th ed. Philadelphia: Wolters Kluwer, Lippincott Williams and Wilkins, 2007. p. 1101-52;
- Meier** KC, Gardner CL, Khoretonenko MV, Klimstra WB, Ryman KD. A mouse model for studying viscerotropic disease caused by yellow fever virus infection. *PLoS Pathog*. 2009 Oct;5(10):e1000614;
- Monath** et al. A live, attenuated recombinant West Nile virus vaccine. *Proc. Natl. Acad. Sci. USA*, 2006. 103: 6694–6699;
- Pugachev** et al. High fidelity of yellow fever virus RNA polymerase. *J Virol*. 2004 Jan;78(2):1032-8;
- Pugachev** KV, Guirakhoo F, Trent DW, Monath TP. Chimeric vaccines against Japanese encephalitis, dengue, and West Nile. In: Levine MM, Dougan G, Good MF, Liu MA, Nabel GJ, Nataro JP, Rappuoli R, editors. *New generation vaccines*, 4th ed. New York: Informa Healthcare USA, 2010, p. 557–69;
- Rice** et al., 1985, *Science*, 229: 726-733;
- Rossi** SL, Tesh RB, Azar SR, Muruato AE, Hanley KA, Auguste AJ, Langsjoen RM, Paessler S, Vasilakis N, Weaver SC. Characterization of a Novel Murine Model to Study Zika Virus. *Am J Trop Med Hyg*. 2016 Jun 1;94(6):1362-9;
- Rumyantsev** et al. Direct random insertion of an influenza virus immunologic determinant into the NS1 glycoprotein of a vaccine flavivirus. *Virology*. 2010 Jan 20;396(2):329-38.
- Rumyantsev** et al., Characterization of the RepliVax platform for replication-defective flavivirus vaccines. *Vaccine*, 2011. 29(32): 5184-94;
- Rumyantsev** et al., Single-dose vaccine against tick-borne encephalitis. *Proc Natl Acad Sci U S A*, 2013. 110(32):13103-8;
- Theiler M. and Smith H.H.**, 1937, *J. Exp. Med.*, 65. 767-786;
- Timiryasova**, T.M. et al., *Am. J. Trop. Med. Hyg.* (2013), vol. 88(5), 962-970 ;
- Zompi** S, Harris E. Animal models of dengue virus infection. *Viruses*. 2012 Jan;4(1):62-82

CLAIMS

1. A live attenuated chimeric Zika virus, comprising a yellow fever virus (YFV) genome whose prM-E sequences have been substituted with sequences coding for the membrane precursor (prM) and envelope (E) proteins of a Zika virus (ZIKV).
2. The live attenuated chimeric Zika virus according to claim 1, wherein the YFV prM-E sequences have been substituted with the prM-E sequences of a Zika virus.
3. The live attenuated chimeric Zika virus of claim 1 or 2, wherein the prM-E sequences of the virus have at least 99% sequence identity to SEQ ID NO: 1, for example 100% sequence identity to SEQ ID NO: 1.
4. The live attenuated chimeric Zika virus according to any one of claims 1 to 3, wherein the sequence of the encoded envelope (E) protein comprises or consists of SEQ ID NO: 4 or comprises or consists of a sequence containing from 1 to 4, for example 1, 2, 3 or 4 mutations compared to SEQ ID NO: 4.
5. The live attenuated chimeric Zika virus according to any one of claims 1 to 3, wherein the sequence of the encoded envelope (E) protein comprises or consists of SEQ ID NO: 4 or comprises or consists of a sequence containing from 1 to 4, for example 1, 2, 3 or 4 mutations compared to SEQ ID NO: 4, but wherein the amino acid at the position corresponding to position 473 of SEQ ID NO: 4 is not mutated with respect to SEQ ID NO: 4.
6. The live attenuated chimeric Zika virus according to any one of claims 1 to 5, wherein the sequence of the encoded prM protein comprises or consists of SEQ ID NO: 3 or comprises or consists of a sequence having from 1 or 4 mutations with respect to SEQ ID NO: 3.
7. The live attenuated chimeric Zika virus according to any one of claims 1 to 6, comprising a mutation in the envelope (E) protein sequence, with respect to SEQ ID NO: 4, at the position corresponding to position 154 of SEQ ID NO: 4.
8. The live attenuated chimeric Zika virus according to any one of claims 1-7, comprising two mutations in the encoded envelope (E) protein sequence, with respect to SEQ ID NO: 4, at the positions corresponding to positions 319 and 443 of SEQ ID NO: 4.
9. The live attenuated chimeric Zika virus according to any one of claims 1-8 comprising a mutation in the encoded envelope (E) protein sequence, with respect to SEQ ID NO: 4, at the position corresponding to position 107 of SEQ ID NO: 4.

10. The live attenuated chimeric Zika virus according to any one of claims 1 to 9, comprising a mutation in the encoded membrane (M) protein sequence at the position which corresponds to position 60 of SEQ ID NO: 5.
11. The live attenuated chimeric Zika virus according to any of claims 1 to 10, wherein the yellow fever virus is derived from an attenuated yellow fever strain.
12. The live attenuated chimeric Zika virus according to Claim 11, wherein the attenuated yellow fever strain is 17D.
13. The live attenuated chimeric Zika virus according to any one of claims 1 to 12, comprising a 3 amino acid deletion in the capsid (C) protein sequence at the positions which correspond to positions 40 to 42 of SEQ ID NO: 7.
14. The live attenuated chimeric Zika virus according to any one of claims 1 to 13, wherein the genome comprises a 5 nucleic acid deletion in the 3'UTR sequence at the position which corresponds to positions 256 to 260 of SEQ ID NO: 8.
15. The live attenuated chimeric Zika virus according to any one of claims 1 to 14, wherein the genomic sequence encoding the C-prM signal sequence codes for a hybrid yellow fever–Zika C-prM signal sequence.
16. The live attenuated chimeric Zika virus according to claim 15, wherein the amino acid sequence of the hybrid yellow fever–Zika C-prM signal sequence is SEQ ID NO: 6 or a sequence having one mutation with respect to SEQ ID NO: 6.
17. An immunogenic composition comprising a live attenuated chimeric Zika virus according to any one of claims 1 to 16, and a pharmaceutically acceptable carrier.
18. A recombinant nucleic acid molecule which comprises a DNA sequence having at least 90% sequence identity to SEQ ID NO: 10, provided the sequence comprises a sub-sequence having at least 99% sequence identity to SEQ ID NO: 10 between the nucleotides 482 to 2497, or a recombinant nucleic acid molecule which comprises the equivalent RNA sequence thereof.
19. A vector comprising the recombinant nucleic acid of claim 18, wherein the recombinant nucleic acid is operably linked to a promoter.

20. A method for producing a live attenuated chimeric Zika virus according to any of claims 1 to 16, said method comprising:
- a) infecting mammalian cells or mosquito cells with a live attenuated chimeric Zika virus according to any one of claims 1 to 16, or transfecting the cells with RNA transcribed from a recombinant nucleic acid according to claim 18, or with a vector according to claim 19;
 - b) cultivating the infected cells in a suitable medium to propagate the live attenuated chimeric Zika virus; and
 - c) harvesting the live attenuated chimeric Zika virus.
21. The method of claim 20, wherein the cells are mammalian cells, preferably VERO cells.
22. The method of claims 20 or 21, wherein step a) is carried out by transfecting the cells with the RNA obtained from the recombinant nucleic acid according to claim 18.
23. The method according to any of claims 20 to 22, wherein the live attenuated chimeric Zika virus comprises a genomic sequence encoding a hybrid yellow fever – Zika C-prM signal sequence having the sequence of SEQ ID NO: 6.
24. The immunogenic composition according to claim 17, for use in a method of inducing a neutralizing antibody response against Zika virus in a mammal, wherein said method comprises administering said immunogenic composition to said mammal and wherein the immunogenic composition is to be administered as a primary vaccination or as a booster vaccination, and optionally wherein the mammal is Zika naïve and dengue naïve.

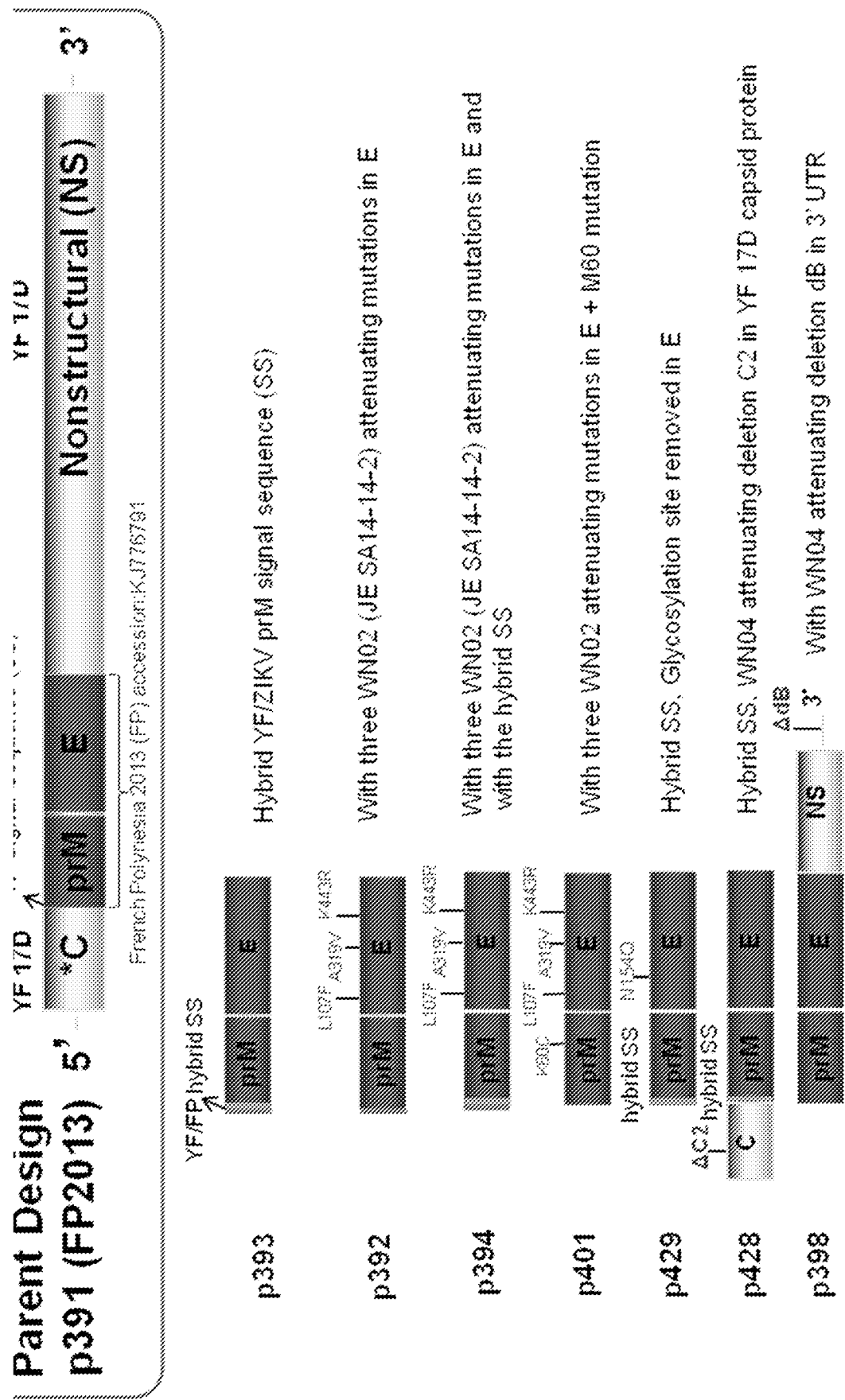


FIG. 1

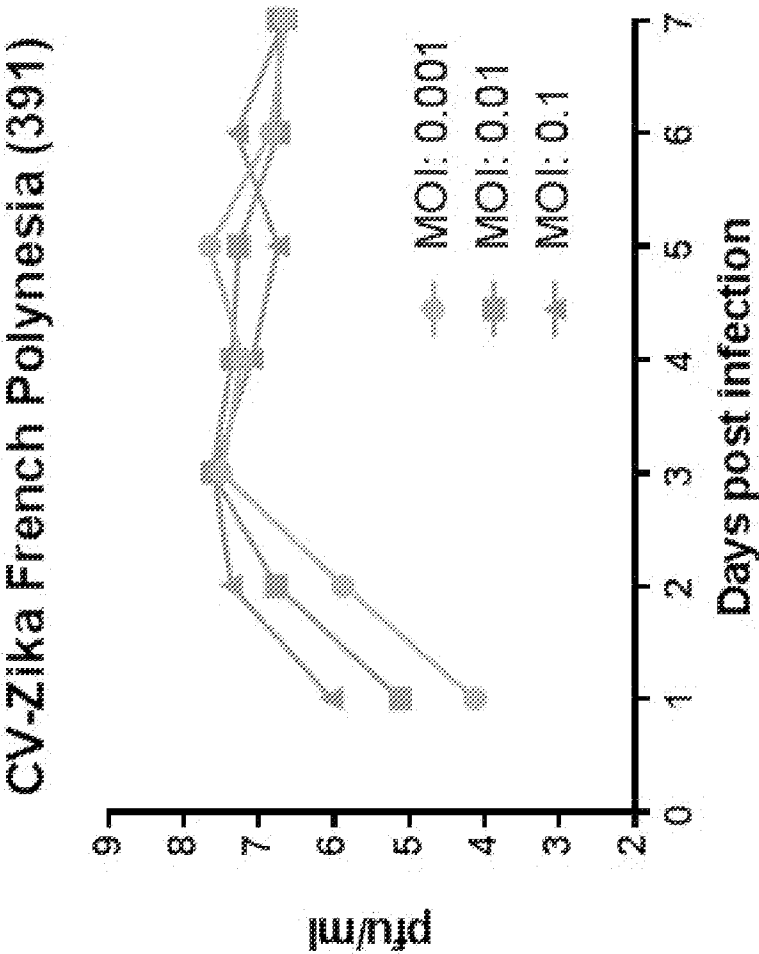


Fig.2c

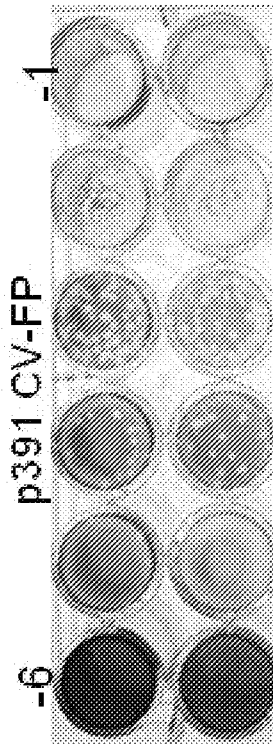


Fig.2a

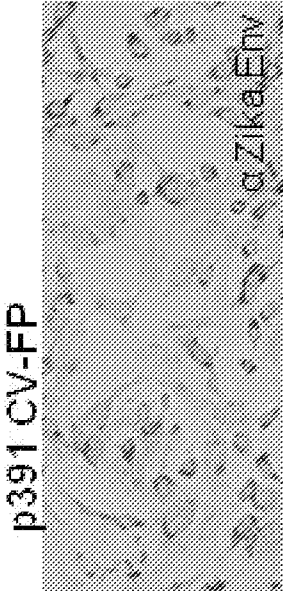


Fig.2b

FIG. 2

CV-Zika French Polynesia Hybrid SS (393)

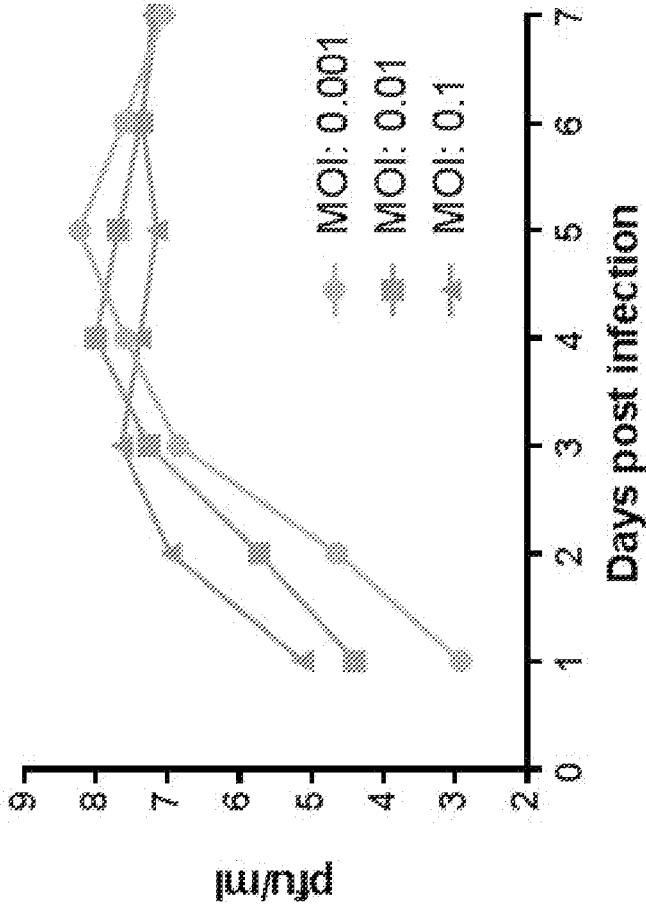


Fig.3c

FIG. 3

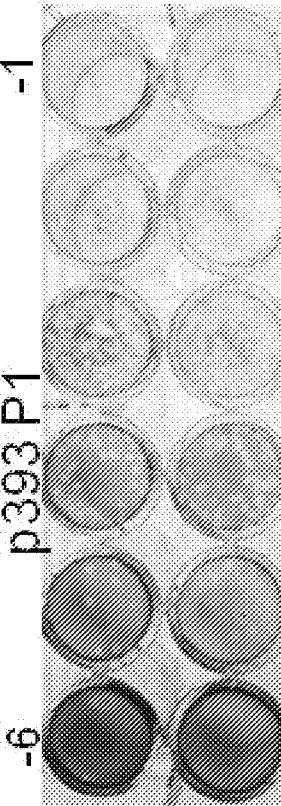


Fig.3a

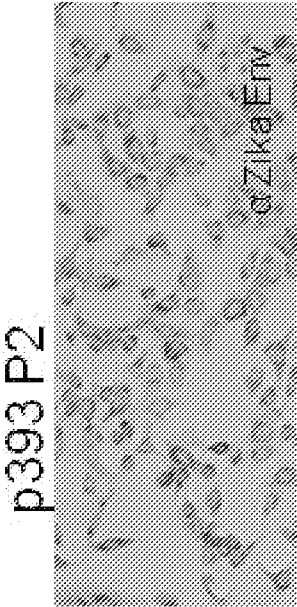


Fig.3b

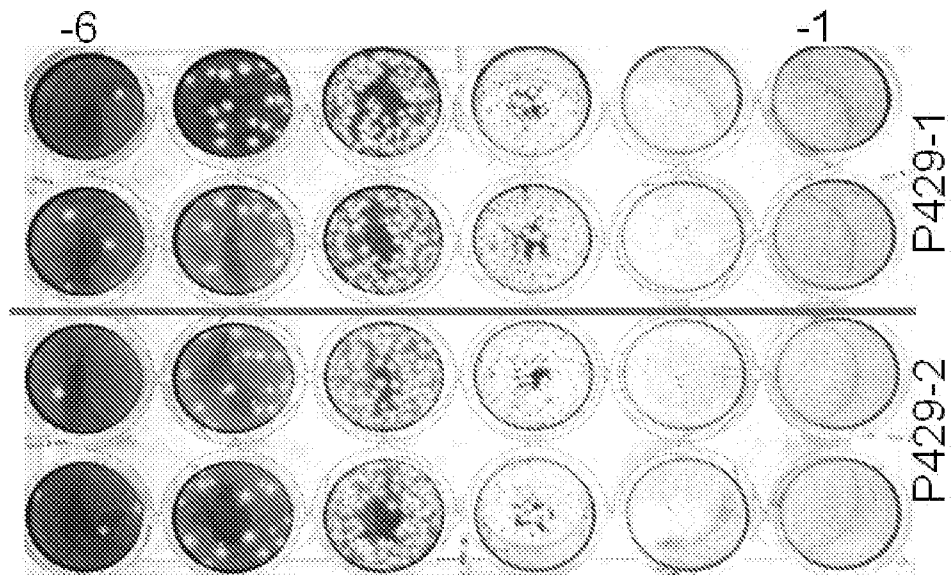


Fig.4a

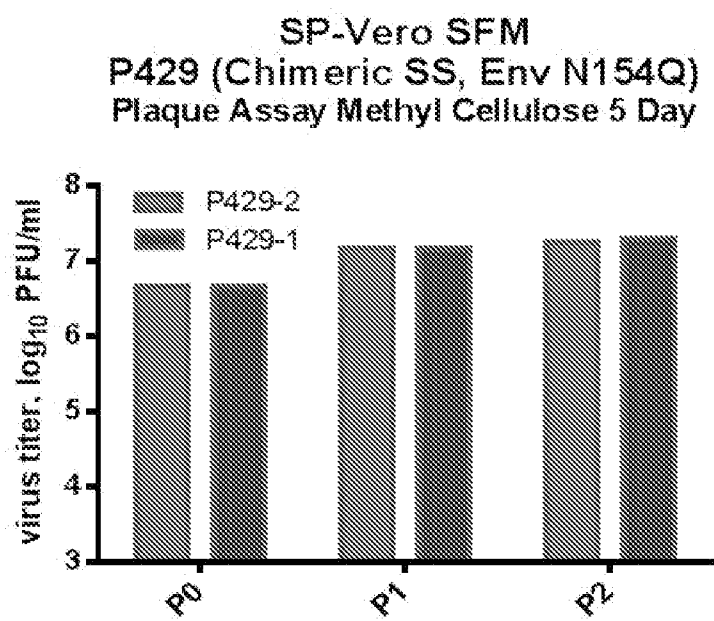


Fig.4b

FIG. 4

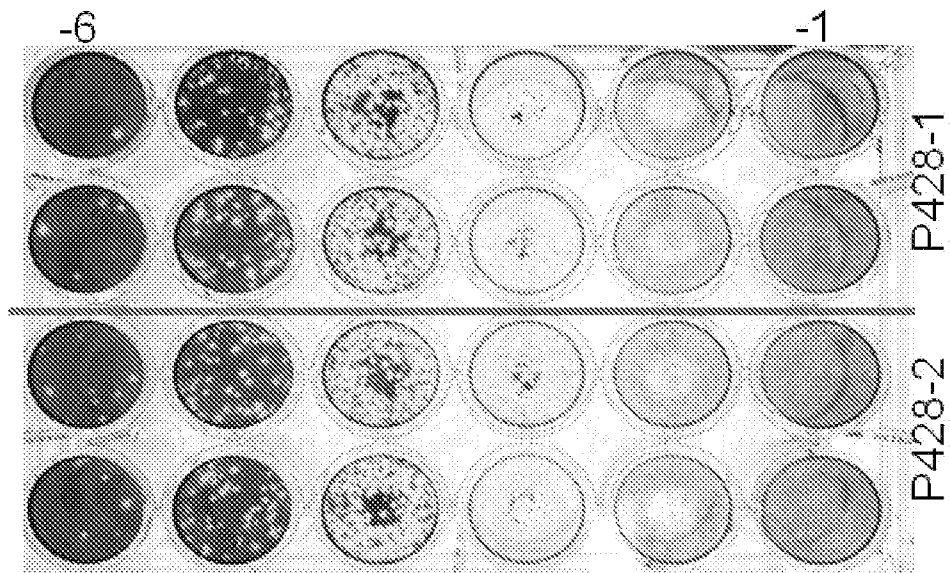


Fig.5a

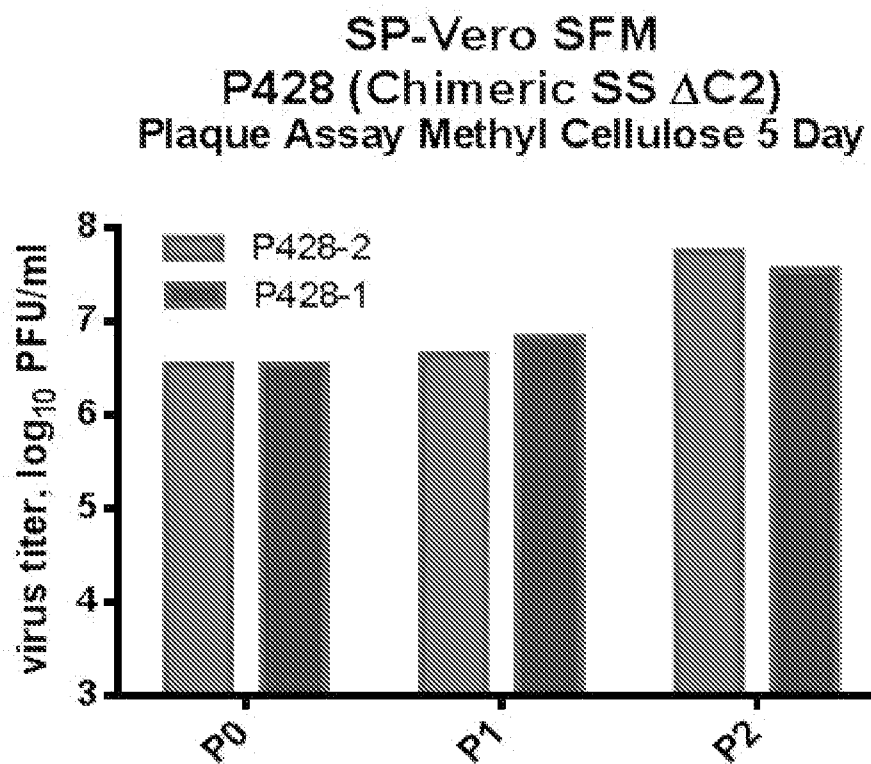


Fig.5b

FIG. 5

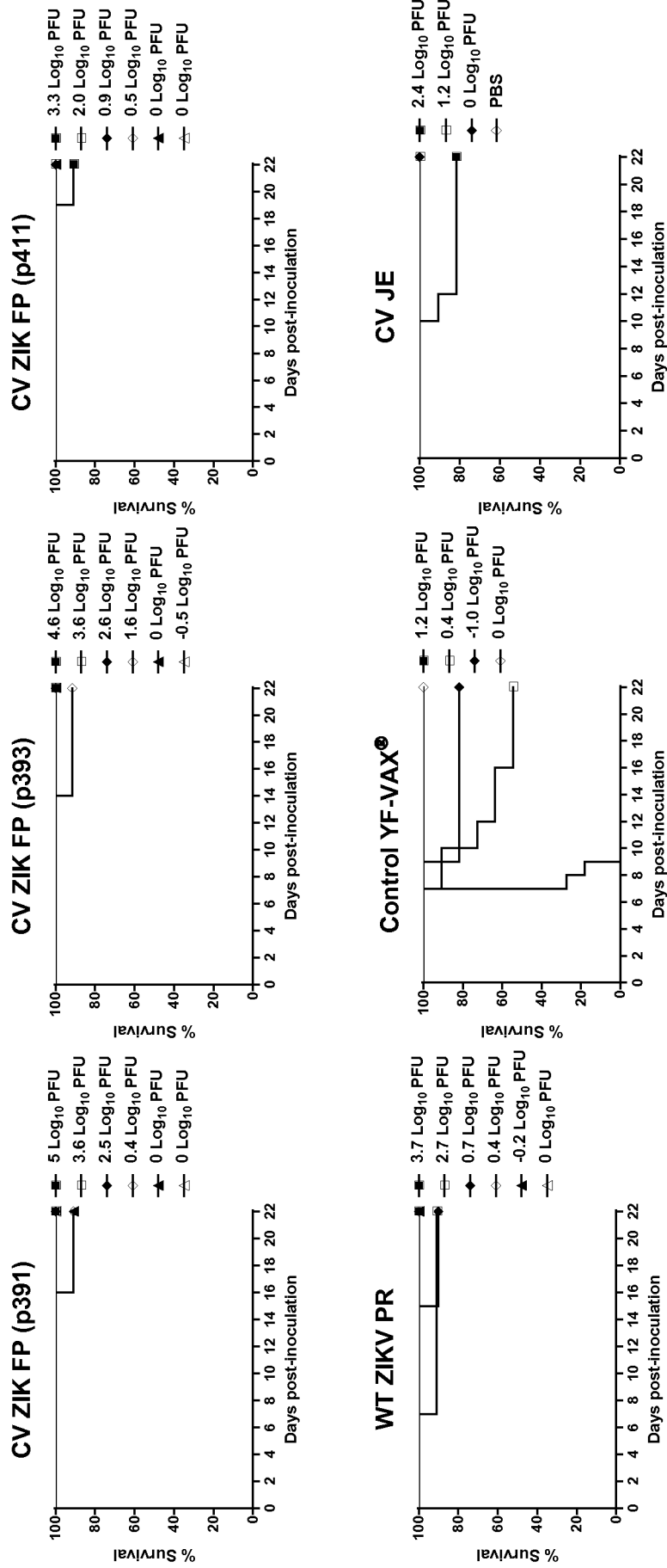


FIG. 6

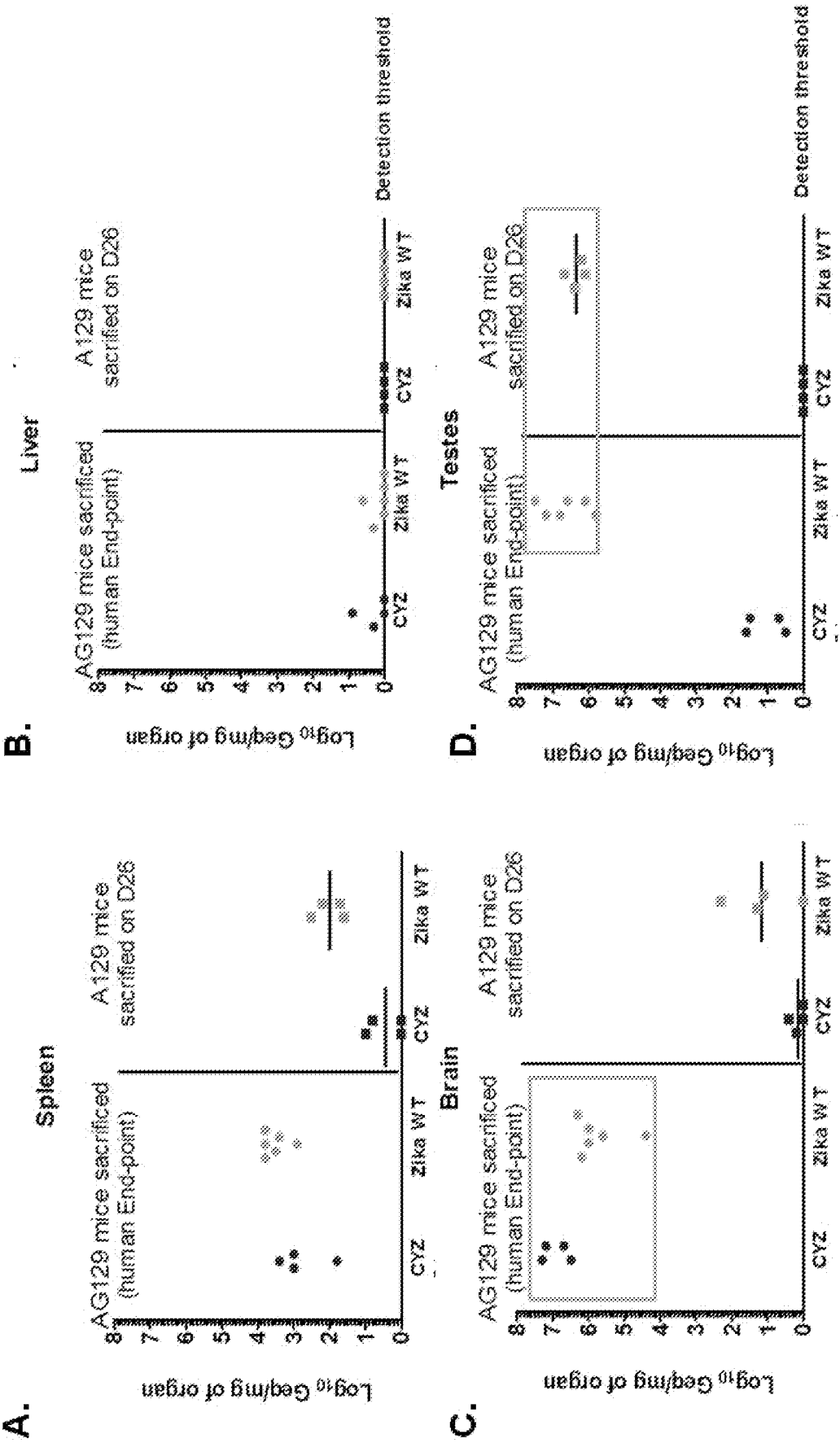
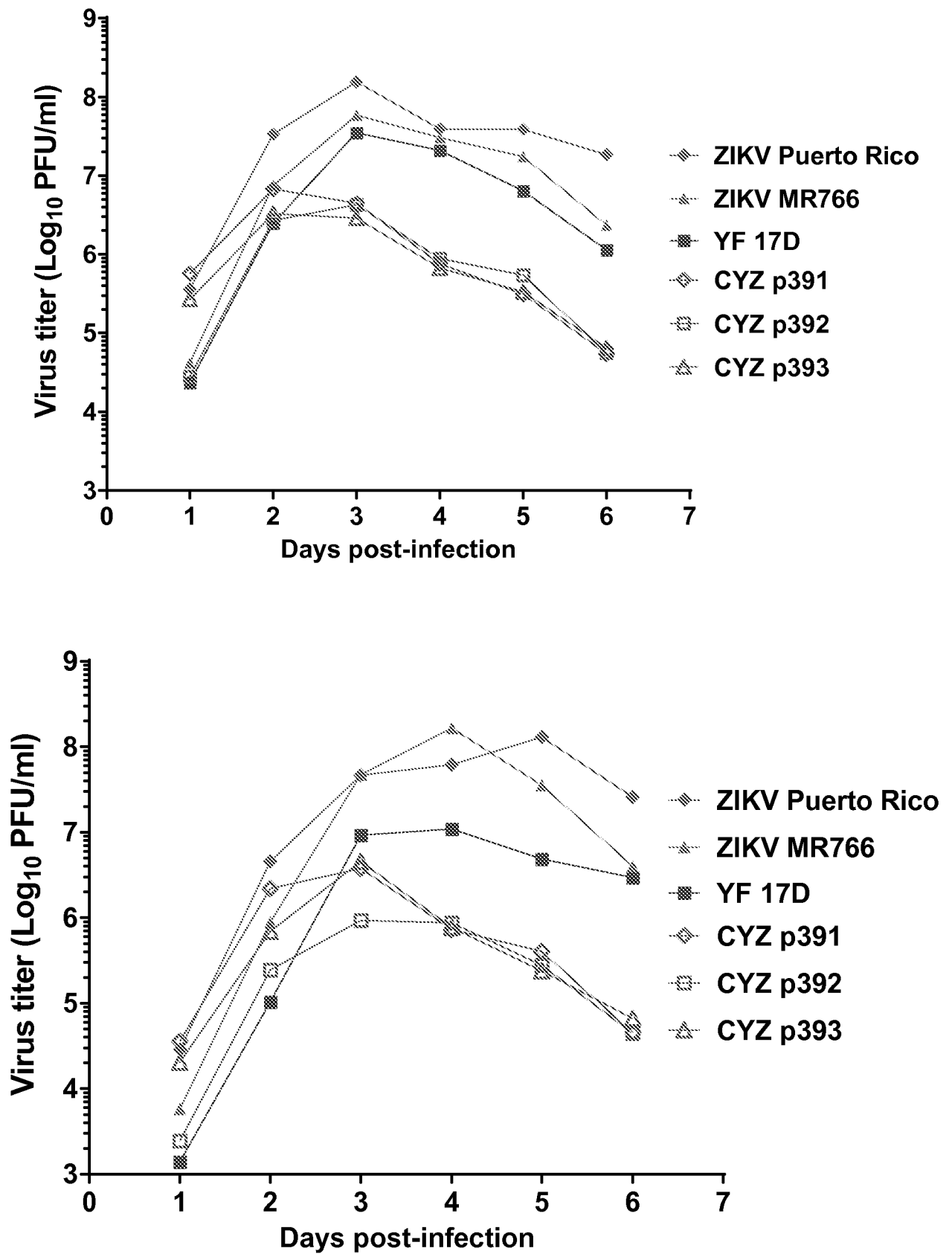
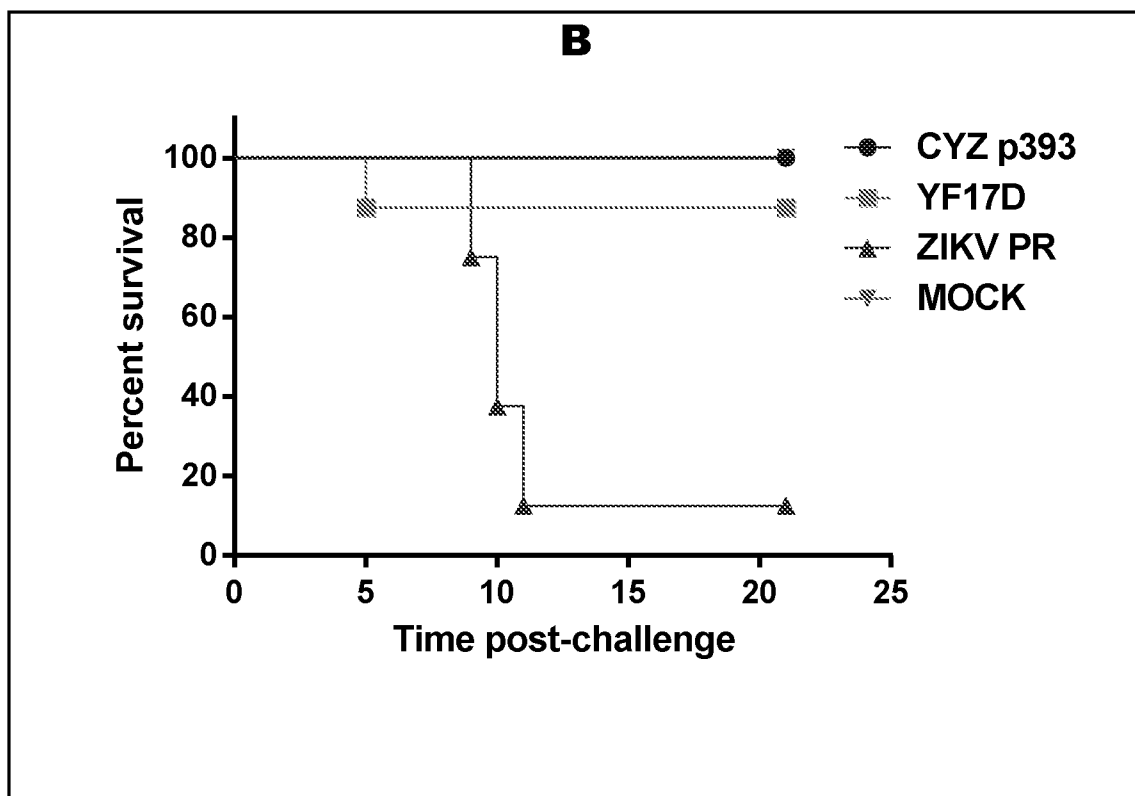
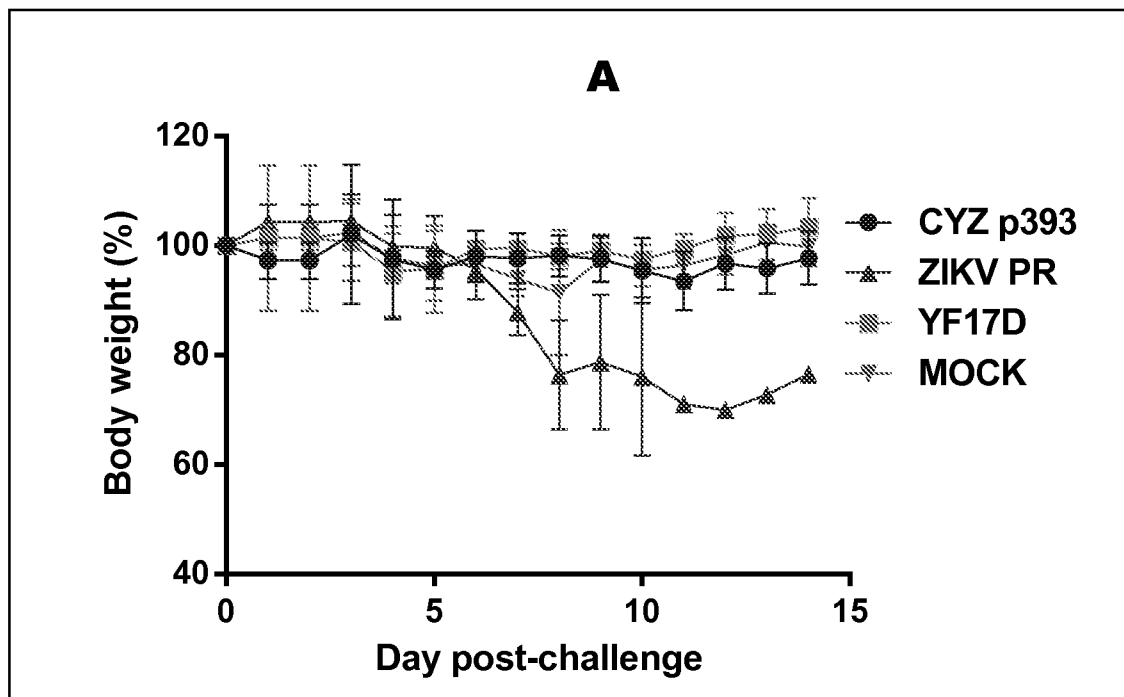


FIG. 7

**FIG. 8**

**FIG. 9**

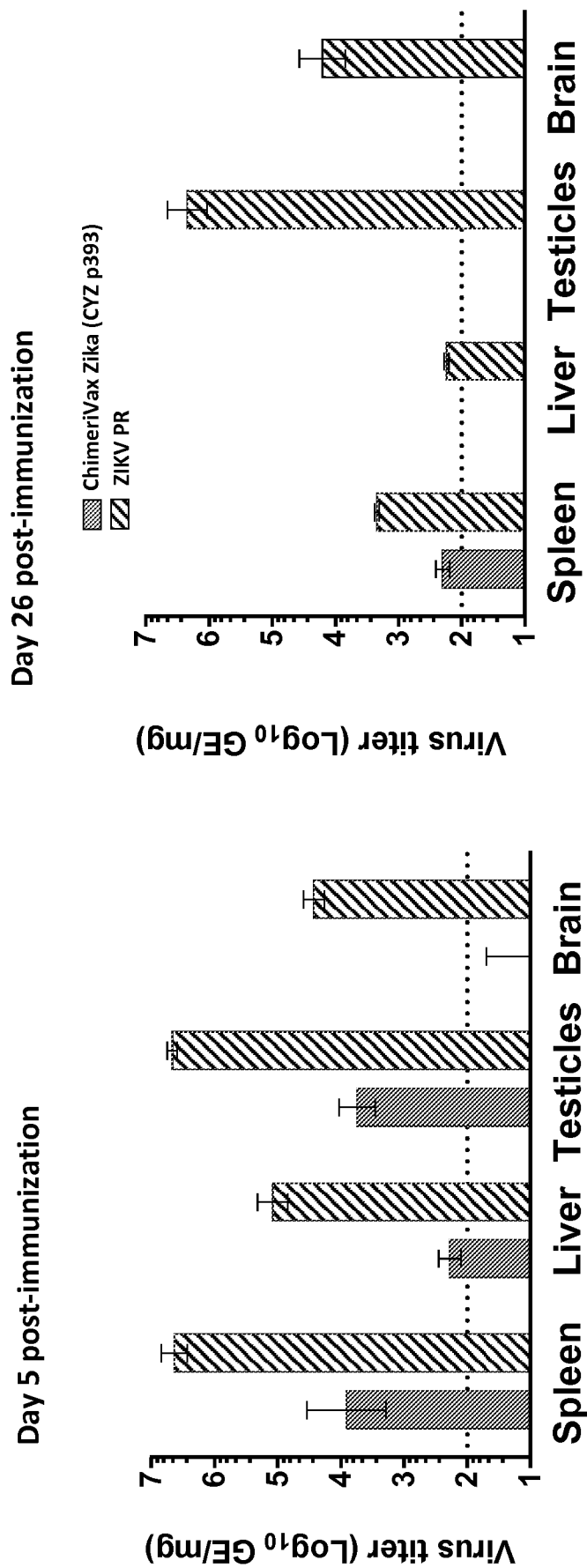


FIG. 10

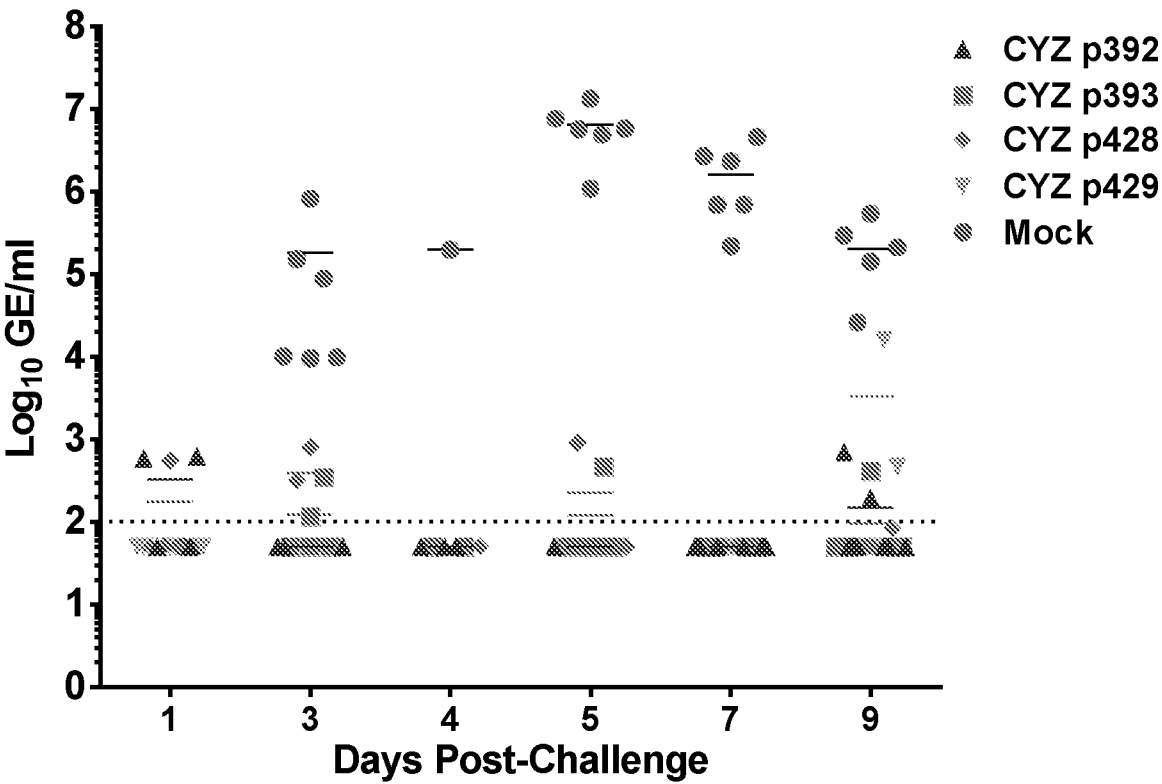


FIG. 11

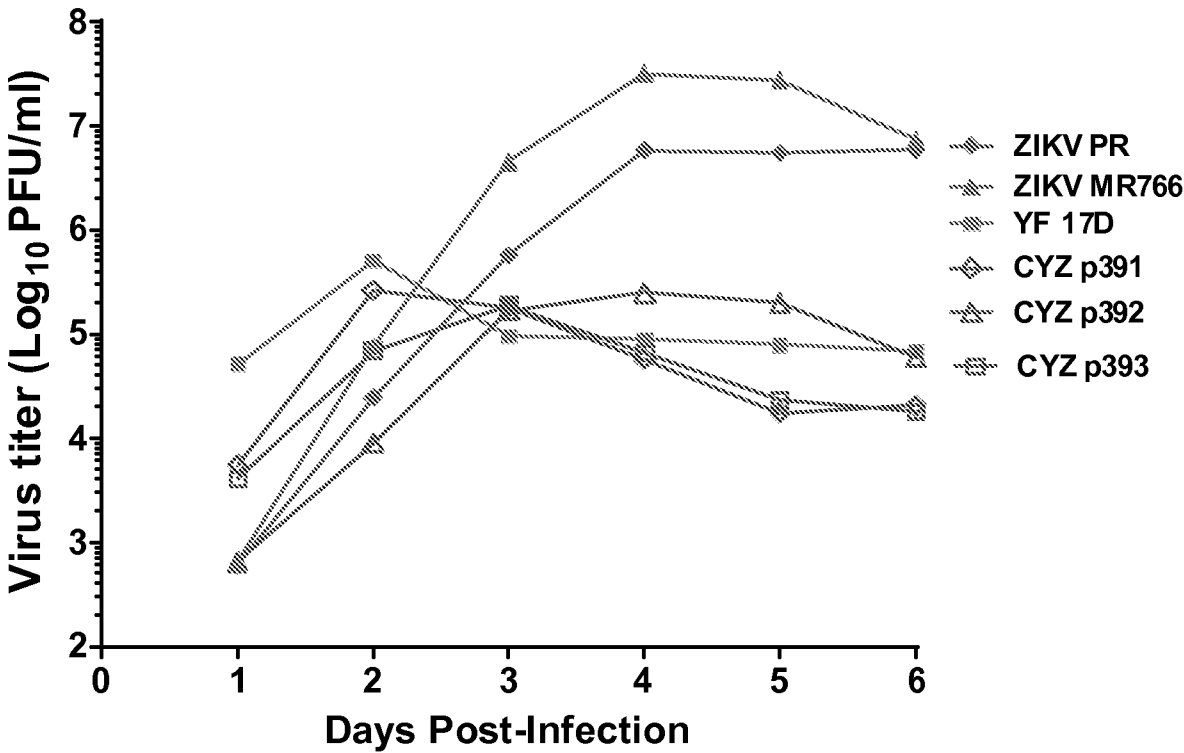
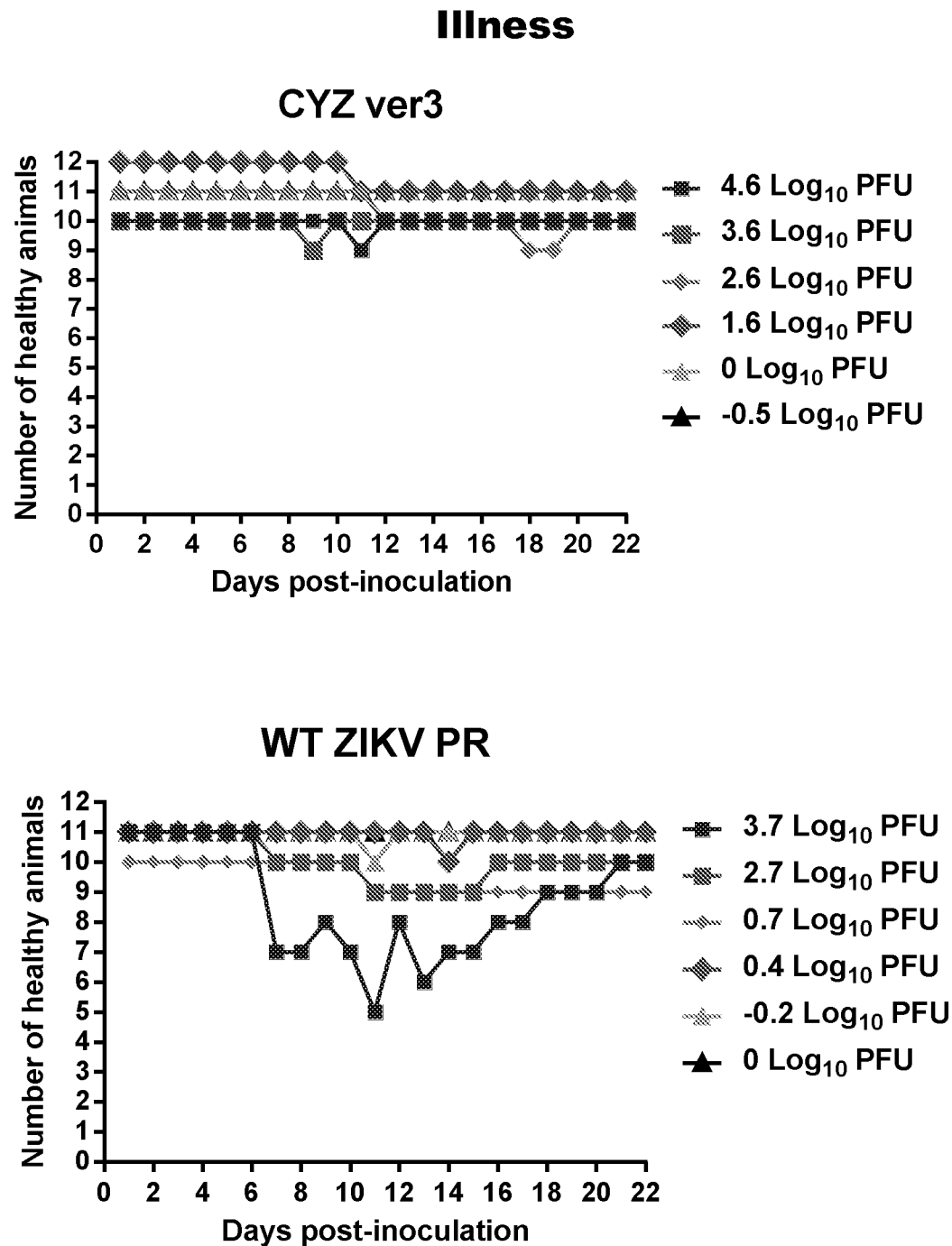


FIG. 12

**FIG. 13**

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2017/001361

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K39/12
ADD.

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

B. FIELDS SEARCHED

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

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, Sequence Search, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ARROYO JUAN ET AL: "ChimeriVax-West Nile virus live-attenuated vaccine: preclinical evaluation of safety, immunogenicity, and efficacy", JOURNAL OF VIROLOGY, THE AMERICAN SOCIETY FOR MICROBIOLOGY, US, vol. 78, no. 22, 1 November 2004 (2004-11-01), pages 12497-12507, XP002528946, ISSN: 0022-538X, DOI: 10.1128/JVI.78.22.12497-12507.2004 cited in the application Discussion; tables 1,5,6	1-24
Y	EP 1 874 346 A1 (ACAMBIS INC [US]) 9 January 2008 (2008-01-09) paragraph [0011]; claim 1 ----- -/-	1-24



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search

18 January 2018

Date of mailing of the international search report

30/01/2018

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer

Renggli-Zulliger, N

INTERNATIONAL SEARCH REPORT

International application No

PCT/IB2017/001361

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GUY B ET AL: "Preclinical and clinical development of YFV 17D-based chimeric vaccines against dengue, West Nile and Japanese encephalitis viruses", VACCINE, ELSEVIER, AMSTERDAM, NL, vol. 28, no. 3, 8 January 2010 (2010-01-08), pages 632-649, XP026793731, ISSN: 0264-410X [retrieved on 2009-10-04] figure 1; table 1 -----	1-24
Y	D. W. C. BEASLEY ET AL: "Envelope Protein Glycosylation Status Influences Mouse Neuroinvasion Phenotype of Genetic Lineage 1 West Nile Virus Strains", JOURNAL OF VIROLOGY., vol. 79, no. 13, 1 July 2005 (2005-07-01), pages 8339-8347, XP055441210, US ISSN: 0022-538X, DOI: 10.1128/JVI.79.13.8339-8347.2005 table 4 -----	1-24
Y	AMIT KUMAR GUPTA ET AL: "ZikaVR: An Integrated Zika Virus Resource for Genomics, Proteomics, Phylogenetic and Therapeutic Analysis", SCIENTIFIC REPORTS, vol. 6, 16 September 2016 (2016-09-16), page 32713, XP055330324, DOI: 10.1038/srep32713 page 5 - page 6 -----	1-24
Y	EP 1 454 988 A1 (INST NAT SANTE ETDE LA RECH ME [FR]) 8 September 2004 (2004-09-08) example 1 -----	1-24
Y	C. BARONTI ET AL: "Complete Coding Sequence of Zika Virus from a French Polynesia Outbreak in 2013", GENOME ANNOUNCEMENTS, vol. 2, no. 3, 5 June 2014 (2014-06-05), pages e00500-14, XP055313805, DOI: 10.1128/genomeA.00500-14 the whole document -----	1-24
Y	BRIAN E DAWES ET AL: "Research and development of Zika virus vaccines", NPJ VACCINES, vol. 1, 28 July 2016 (2016-07-28), page 16007, XP055331389, DOI: 10.1038/npjvaccines.2016.7 page 4, left-hand column, paragraph 3; table 1 -----	1-24

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/IB2017/001361

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 1874346	A1	09-01-2008	AU 2006239954 A1 02-11-2006 BR PI0609949 A2 11-05-2010 CA 2605924 A1 02-11-2006 CN 103031279 A 10-04-2013 EP 1874346 A1 09-01-2008 ES 2478303 T3 21-07-2014 JP 5469336 B2 16-04-2014 JP 2008538698 A 06-11-2008 US 2008175862 A1 24-07-2008 US 2012288520 A1 15-11-2012 WO 2006116182 A1 02-11-2006
EP 1454988	A1	08-09-2004	CA 2517601 A1 16-09-2004 EP 1454988 A1 08-09-2004 EP 1599592 A1 30-11-2005 JP 2007527205 A 27-09-2007 WO 2004078986 A1 16-09-2004