

FORM 2

THE PATENTS ACT, 1970
(39 of 1970)
&
THE PATENTS RULES, 2006

COMPLETE SPECIFICATION
(See Section 10 and rule 13)

“VACCINE COMPOSITIONS”

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The following specification particularly describes the invention and the manner
in which it is to be performed:

5 FIELD OF THE INVENTION

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The present invention discloses vaccine compositions comprising Zika virus antigens for prophylaxis and treatment of Zika virus infections in mammals. The invention also discloses stable vaccine compositions comprising Zika virus antigens with one or more arbovirus antigens such as Chikungunya and/ or Japanese encephalitis virus antigens. The present invention also relates to the methods of preparation, formulation and use of the same for simultaneously eliciting immune response to each of the above mentioned pathogens in mammals, and suitable for immunizing human subjects

15 BACKGROUND OF THE INVENTION

At present there is no vaccine available in the world for prophylaxis or treatment against Zika virus infections. Therefore, there is no prior art relevant to the invention disclosed in this application. However, for general understanding of the background and objectives behind this invention is discussed hereinafter in below paragraphs.

The Inventors of this patent application anticipated the epidemic potential of Zika virus in regions with high prevalence of *Aedes* mosquitoes, particularly *Aedes aegypti* that transmits the virus. The interest in initiating the Zika vaccine project early on, several months before the causal link of Zika virus infection to Guillain Barre Syndrome and to microcephaly became public knowledge in December 2015, was that there was no preparedness in any country in the world, nor measures initiated by anyone at that time to develop a vaccine to stop the ongoing virus transmission in countries such as Brazil, and to prevent further transmission in countries at risk for Zika virus. Increased International travel to and from regions with ongoing virus transmission impose a major risk to initiate an outbreak in countries with high prevalence of *Aedes* mosquitoes, particularly *Ae.aegypti* and those having a large naïve population hither to unexposed to the virus. The clinical picture of Zika virus infection in the early stages with characteristic high fever, maculopapular rashes and arthralgia is strikingly similar to the early onset symptoms of Chikungunya and Dengue virus infections that make differential diagnosis particularly challenging.

Zika virus vaccine project was initiated at the time when very little or no information was available on virus pathogenesis, genetic diversity, transmission, diagnosis, serological correlates for protection or animal models to test the vaccine concepts. From vaccine point of view, there was no information on whether the virus can be cultured *in vitro* in cell substrates and if yes, which cell substrates are best suitable, mechanism of adaptation to cells, potential virus titers and the feasibility to manufacture the vaccine product for human administration, as the published information at that time pertained to passaging the virus in mouse brain which is not suitable for vaccine production. Bharat Biotech initiated steps to start Zika vaccine project in late 2014, and commenced the experimental work soon thereafter resulting into this said patent application.

Arbovirus (arthropod-borne) infections are caused by viruses that are spread by arthropods such as mosquitoes. They cause significant human illness ranging from mild, asymptomatic infection to acute encephalitis or hemorrhagic fever that can prove fatal. The most significant

arboviruses causing human illness belong to three viral families, *Togaviridae*, *Flaviviridae*, and *Bunyaviridae*. Arbovirus infections are rampant in developing countries and cause severe morbidity particularly in the elderly population. The common characteristic feature of arbovirus infections caused by Dengue, Chikungunya, Zika, Japanese encephalitis and West Nile viruses among others is fever, headache, myalgia, joint pains with swelling and maculopapular rashes during the acute phase of the viral infection. Arthralgia is particularly a characteristic feature of Chikungunya, Dengue and Zika virus fever. Co-infections are common as the arboviruses largely share the same mosquito vectors such as for example Dengue, Chikungunya and Zika viruses that are transmitted by *Aedes* mosquitoes. Japanese encephalitis virus and West Nile viruses are transmitted predominantly by *Culex* mosquitoes. The problem is acute in developing countries where mosquito vector control programs have been ineffective and largely unsuccessful. The problem is compounded by the fact that there are no robust diagnostic methods available for diagnosing the disease causing viruses with certainty. International travel has aided widespread dissemination of these infectious agents, and diseases like Dengue and Chikungunya hitherto confined to tropical countries are now spread geographically to new areas and to temperate regions. Zika virus is reportedly spread to over 65 countries in the last two years. Autochthonous epidemic outbreaks reported in few countries in these regions are sustained by the local population of mosquito vectors.

Zika virus (ZIKV) is an emerging zoonotic arbovirus, belonging to the *Flaviviridae* family. Like Dengue and Chikungunya viruses, Zika virus can also be transmitted by *Aedes* mosquitoes more specifically *A. furcifer*, *A. taylori*, *A. luteocephalus*, *A. africanus*, *A. albopictus* and predominantly by *A. aegypti*. Travel tourism to nations where the recent epidemics were reported such as Polynesia has aided the geographical spread of the virus infection to Brazil, Columbia, Italy and to other countries. An autochthonous outbreak of the virus was reported in Italy caused by the locally established *Aedes* mosquitoes. In Asia, Zika virus infection has occurred sporadically in Cambodia, Thailand, Indonesia, Malaysia and Bangladesh although large epidemic outbreaks have not been reported in these regions.

Chikungunya virus (CHIKV) is an *Alphavirus* of the family *Togaviridae*. The virus causes self-limiting febrile infection characterized by acute onset of high fever, headache, myalgia, arthralgia, swelling in joints and maculopapular rashes. Severe symptoms such as hemorrhagia, fulminant hepatitis and neurological symptoms were reported in the more recent epidemics. Chikungunya virus is transmitted by both the *Aedes aegypti* and *Aedes albopictus* mosquitoes.

Japanese encephalitis virus (JEV) is also a flavivirus of the family *Flaviviridae* and is transmitted largely by the *Culex* mosquitoes. JEV is related to Dengue, Yellow fever virus, Zika and West Nile viruses. JEV infection is largely asymptomatic, but in general it causes malaise with fever, headache and other flu-like symptoms. Rarely, the clinical infection progresses to encephalitis with seizures, spastic paralysis, coma and death. Children are particularly susceptible. In the countries endemic for JEV, most adults have natural immunity after childhood infection. Adults not exposed to the infection during childhood are susceptible at any age. The case-fatality rate in JEV caused by encephalitis can be as high as 30%. Neurological complications or psychiatric sequelae occur in high proportion of the cases with encephalitis. Globally, about 3 billion population is at risk for JEV infection. A few vaccines for prophylaxis of JEV infection have been successfully commercialized.

Dengue virus (DENV) is a member of *Flaviviridae* family. The arbovirus infections can no longer be considered region specific as they are now geographically widespread and are significant public health problem in many parts of the world. The morbidity caused by the aforementioned arbovirus infections is usually high, and arthralgia in particular, adversely impacts physical mobility of the patients. Zika virus causes more serious congenital birth

deformities during infection in pregnancy, and Zika related Guillain Barre syndrome have been confirmed in the ongoing epidemics. Like any other viral infections, no specific therapeutics is available. Prophylactic vaccination can effectively interrupt Zika virus transmission and a vaccine would be the front line of defense from the Zika virus disease.

5 With this in mind, an effective strategy was developed to prevent further transmission of Zika virus to protect naïve population in countries with ongoing epidemics and in countries where active Zika virus transmission has not been reported as yet. A combination vaccine for arbovirus infections is good strategy to protect vulnerable population from debilitating illnesses caused by Dengue, Chikungunya, Zika, Japanese encephalitis, West Nile and
10 Yellow Fever viruses. Vaccines for JEV, Yellow Fever and one for Dengue serotypes has been commercialized and those for West Nile and CHIKV are in clinical development. There is no vaccine for Zika virus infection as yet, and the current invention discloses the methods for development of the first candidate Zika vaccines.

15 However, the choice of antigens to include in such a vaccine kit depends on several factors. The antibody dependent enhancement of virus caused by the Dengue serotypes is well researched and published and so also the cross reactivity of flavivirus antibodies. But what was not clear is that if there would be such interference or cross reactivity of prevalent Chikungunya antibodies in the same population that is affected by Zika virus. Similarly, it
20 was not known if antibodies to Japanese encephalitis virus could cause antigenic interference in developing immunity to Zika virus and it was an interesting thought to study the same. The proposed work also provides an insight to any possible immune interference caused by prevalent JE and CHIKV antibodies to candidate Zika vaccine.

25 In current invention, candidate Zika virus vaccines have been developed and tested for potency with various formulations to elicit the appropriate level of immune response to protect against Zika virus disease. As there was no significant antigenic interference to Zika induced immune response by Japanese encephalitis virus vaccine and Chikungunya virus vaccine when co-administered or combined as a combination vaccine, the formulations were
30 effective in eliciting high level of neutralizing antibodies capable of conferring protection against each of the viruses

OBJECTS OF THE INVENTION

35 One object of the invention is to provide stable immunogenic compositions for prophylaxis and treatment of Zika virus infections.

40 Another object of the invention is to provide methods for adaption and growth of Zika virus in Vero cells.

Another object of the invention is to provide methods for the preparation of inactivated Zika virus vaccine and purification of Zika virus bulk antigen.

45 One more object of the invention is to provide methods for Zika virus inactivation by chemical means with formalin, beta propiolactone and hydrogen peroxide

50 Yet another object of the invention is to provide methods for Zika virus inactivation by physical means such as heat, gamma irradiation and ultraviolet light

Yet another object of the invention is to provide methods for the preparation and formulation of recombinant Zika virus antigens comprising the prME protein and testing for immunogenicity in animals

5 A further object of the invention is to provide methods for formulations of Zika virus antigens with different adjuvants and estimation of immune response to the formulations in animals.

10 Yet another object of the invention is to provide kinetics of immune response to single dose, two and three doses of formalin and BPL inactivated Zika virus vaccine in animals.

One more object of the invention is to provide immunogenic compositions for prophylaxis of Zika and Chikungunya virus infections

15 Another object of the invention is to provide immunogenic compositions for prophylaxis of Zika, Chikungunya and Japanese encephalitis virus infections.

20 SUMMARY OF THE INVENTION

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- The present invention is directed to compositions and methods of manufacture of vaccine formulations for prophylaxis and treatment of Zika virus infections as well as infections caused by other arboviruses such as Chikungunya virus and Japanese encephalitis virus.
- 25 • In one aspect, the invention is directed to vaccine compositions for prophylaxis and treatment of Zika virus infections, wherein the said compositions comprise Zika virus antigens in immunogenic compositions and may also comprise one or more arbovirus antigens such as Chikungunya virus and Japanese encephalitis virus antigens, along with suitable adjuvants and excipients.
- 30 • In another aspect, the invention is directed to a method of obtaining the vaccine formulations by a process which comprises:
 - (a) Using Vero cell line as cell substrate for Zika virus culture
 - (b) Scaling up the Zika virus culture upto a harvest volume of 10L
 - (c) Inactivating the virus culture
 - 35 (d) Purifying the virus culture
 - (e) In another aspect, recombinant cloning and expressing Zika virus pRME protein.
- 40 • In one embodiment of the invention, Vero cell line was used as the cell substrate for culture of Zika virus and was grown in culture medium with or without the use of serum.
- In another embodiment of the invention, the Zika virus was adapted by repeated serial passage in Vero cells to obtain higher titers.
- In another embodiment of the invention, Zika virus was passaged in C6/36 Ae. Albopictus cells followed by growth in Vero cells to increase the titer.
- 45 • In another embodiment of the invention, processes for scaling up the virus culture and further purifying the scaled up virus cultures is disclosed, wherein the harvest volume was about 8-10 L. The viral harvest was inactivated using various methods. The virus was then purified.

- In another embodiment of the invention, inactivation method is selected from a group of Formalin inactivation, Beta Propiolactone (BPL) inactivation, heat inactivation, UV inactivation, gamma inactivation, in the presence or absence of virus stabilizing agents and amino acids.
- 5 • In a preferred embodiment of the invention, amino acids were selected individually or in combination, from a group L-Histidine, L-Glutamic acid, L-Glycine and L-Aspartic acid and L-Glutamine and human serum albumin.
- In another preferred embodiment of this invention, the purification method is selected by use of cellulose sulphate, DEAE-Sephadex CM-sephadex with salt gradient, by gel
10 filtration on Captopore-700, Sepharose CL-4B, ceramic hydroxyapatite column with gradient of 0.2M to 0.8M phosphate followed by diafiltration, and ultracentrifugation on a 20-60% sucrose gradient, most preferably by Captopore 700 column.
- Another embodiment of the invention is directed to recombinant cloning and expression of Zika virus prME protein is provided. The method of recombinant
15 cloning utilizes a site specific transposition of the expression cassette with the cloned inserts into a baculovirus shuttle vector propagated in *E.coli* and expressed in insect cells.
- In another embodiment of the invention, vaccine formulations are provided. The vaccine may comprise of one or more Arbovirus antigens selected from Zika virus, Chikungunya virus and Japanese encephalitis viruses.
- 20 • In another embodiment, adjuvants can be selected from a group of aluminium salts, inulin, alginate, combination of inulin and aluminium hydroxide, monophosphoryl lipid A (MPL), resiquimod, muramyl dipeptide (MDP), N-glycolyl dipeptide (GMDP), poly IC, CpG oligonucleotide, resiquimod, aluminium hydroxide with MPL, any water in oil emulsion, any oil in water emulsion that contains one or
25 more of the following constituents: squalene or its analogues or any pharmaceutically acceptable oil, tween-80, sorbitan trioleate, alpha-tocopherol, cholecalciferol or any of the analogues and derivatives of the molecules thereof, or calcium phosphate or any combination of the adjuvants.
- In another embodiment of the invention, the formulations is prepared with excipients and preservatives.
- In another embodiment of the invention, stabilizing agents in the vaccine formulation were used individually or in combinations of sorbitol, L-glycine, mannitol, L-glutamic acid and human serum albumin in various concentration was used to study
35 the same.
- In another embodiment of the invention, the potency of the vaccine formulations have been tested in animal models to show complete protection from viremia over a wide range of dosage.
- In another embodiment of the invention, the combination vaccine formulations were also effective in providing adequate protection against Japanese Encephalitis as well
40 as Chikungunya viruses.
- In another embodiment of the invention, the antisera confers passive immunity in rabbit against Zika virus infection to offer complete protection against viremia while viremia was detected in the control animals that persisted up to 6 days after virus
45 challenge.
- In another embodiment of the invention, the candidate inactivated Zika virus vaccine can be administered either as a single dose, or in two or more doses by intramuscular route

- In another embodiment of the invention, assays for neutralizing antibody titers were conducted to check the antibody levels against vaccine formulations of the present invention which has shown to elicit high level of neutralizing antibodies.
- 5 • In another embodiment of the invention, cross neutralization studies exhibited that inactivated vaccine formulations of the present invention would be equally protective and potent against any Zika virus strain.
- In another preferred embodiment of the invention, antibody titers to both BPL inactivated and formalin inactivated Zika vaccine formulations were higher with aluminium hydroxide than with antigens alone.
- 10 • In another embodiment of the invention, quality of antibody responses to the vaccine formulations of the present invention by antibody avidity assays indicated that high affinity antibody were developed over time with booster doses.

15 Accordingly, the invention provides a stable vaccine composition comprising one or more arbovirus antigens selected from Zika virus, Chikungunya virus and Japanese encephalitis virus, said antigens being formulated with or without an adjuvant in pharmaceutically acceptable buffer, wherein the vaccine composition elicits protective immune response to each of the viruses in mammals. The Zika virus antigen of the composition is effective for treatment, diagnosis and prophylaxis against any genotype/genotypic variants/strains of Zika virus, wherein the composition is effective against any genotype/genotypic variants/strains/synthetic Zika viruses that share anywhere between 50% to 100% identity at the amino acid level in any region of the genome. The composition of the invention comprises Zika virus antigens of any genotype/genotypic variant/strains/synthetic Zika virus, wherein the antibodies against any of the aforementioned Zika virus types cross neutralizes the homologous virus or any heterologous Zika virus strain that shares at least 50% -100% amino acid identity in any region of its whole genome, particularly the envelope E protein.

25 The antigens of Zika virus, Chikungunya virus and Japanese encephalitis virus of the composition are inactivated whole virion (virus) antigens. Whereas, the Zika and Chikungunya virus antigens are purified recombinant antigens.

30 The Zika virus antigen of the invention is prepared using Vero cells as cell substrate by adapting the virus to Vero cells.

35 The Zika virus antigen of the composition of the invention is a purified and concentrated antigen obtained from one or more methods selected from:

- a. ultracentrifugation;
- b. density gradient centrifugation;
- 40 c. clarification of the viral harvest using membrane filtration, followed by purification by column chromatography; and
- d. tangential flow filtration using membranes with cut off from 100 kDa to 300 kDa, wherein tangential filtration is carried out either before or after virus inactivation.

45 Wherein the purification by column chromatography comprises gel filtration, mixed mode resin column chromatography, ion exchange column chromatography, affinity matrix chromatography and hydrophobic interaction chromatography. The column chromatography elutes majority of the virus antigen in the flow through such as Capto Core 700, most preferably Capto Core 700 wherein the virus sample is purified on Capto Core 700 column and is eluted in the flow through.

The Zika virus of the composition is inactivated by at least one or more of a chemical inactivating agent, a physical inactivating agent and an irradiating agent, wherein the inactivation of Zika virus is carried out before or after purification of the virus. In an exemplary embodiment the Zika virus is inactivated by chemical inactivating agent selected from formalin (formaldehyde), beta propiolactone (BPL) and hydrogen peroxide.

In one preferred embodiment the Zika virus is inactivated by any one of the following methods selected from:

- a. Formalin treatment at any concentration ranging from 1: 500 up to 1: 4000 v/v of formalin: virus, at 8⁰C to 37⁰C, preferably 25±3⁰C, for at least 1 to 7 days;
- b. Formalin treatment at any concentration ranging from 1:500 up to 1: 4000 v/v of formalin: virus, at 2⁰C to 8⁰C for at least 10 to 30 days;
- c. Beta propiolactone (henceforth BPL) at any concentration ranging from 1:500 up to 1: 4000 v/v of BPL: virus, for at least 24 to 48 hrs at temperatures ranging from 8⁰C to 30⁰C, preferably 25±3⁰C, for 48 hours;
- d. Beta propiolactone at any concentration ranging from 1: 500 up to 1:4000 (BPL: virus, v/v), at 2⁰C to 8⁰C for at least 3-7 days;
- e. A combination of BPL and formalin at any of the aforementioned conditions, preferably BPL inactivation at 1:3000 (BPL :virus, v/v) for 24 hours followed by formalin inactivation at 1: 3000 (formalin: virus, v/v) for 24to 48 hours at 15⁰C to 30⁰C, preferably 25±3⁰C ;
- f. Hydrogen peroxide at any concentration from 0.1 to 3%, preferably 0.1 to 1% at any temperature from 20 - 30⁰C for 5 minutes to 120 minutes.

In one embodiment, the inactivation of the Zika virus by irradiating agent comprises inactivation by gamma irradiation by exposure from 20 kGy (Kilo Gray) up to 35 kGy, preferably 25 kGy to 30 kGy from a ⁶⁰Co source.

In another embodiment, the inactivation of the Zika virus by irradiating agent comprises inactivation by UV irradiation by exposure to 254 nm for 30 – 60 minutes.

In a further embodiment, the virus is inactivated by heat treatment at a temperature between 50⁰C to 65⁰C for 30 min up to 2 hrs.

The buffer used in the invention may be selected from the list comprising of phosphate buffer, citrate buffer, phosphate citrate buffer, borate buffer, tris(hydroxymethyl)aminomethane (Tris) containing buffer, succinate buffer, buffers containing glycine or histidine as one of the buffering agents, wherein phosphate buffer is sodium phosphate buffer at concentration of 5 mM up to 200 mM of phosphate ions of any pH between 6.50 to pH 9, and optionally containing sodium chloride at a concentration of 50 to 200 mM. The buffer maintains the pH in a liquid composition above pH 6.5, preferably above pH 7.0 throughout the bioprocess from viral culture up to preparation of purified inactivated virus bulk.

In one embodiment, the inactivation of Zika virus is carried out in the presence of a stabilizing agent selected from lactose, sucrose, trehalose, maltose, mannose, iso-maltose, raffinose, stachyose, lactobiose, sorbitol, mannitol, lactobionic acid, dextran, L-glycine, L-histidine, L-glutamic acid, L-aspartic acid and human serum albumin or combinations thereof. However, in one preferred embodiment, the stabilizing agent may be selected from:

- a. 2% sorbitol and 1% L-glycine;

- b. 1% sorbitol and 0.5 % L-glycine;
- c. 1% mannitol and 0.5% L-glycine;
- d. 1% mannitol and 0.5% L-glutamic acid; and
- e. 1% sorbitol and 0.5% L-glycine, 1% human serum albumin.

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In an exemplary embodiment, the inactivation of Zika virus comprises inactivation of any genotype/strain, live attenuated Zika virus, deactivated virus, virus like particles, chimeric virus particles that carry any Zika virus antigens particularly the E protein in any heterologous virus backbone, in vectored vaccines and infectious synthetic virus particles derived *in vitro* or *in vivo* using the sequence of any Zika virus genome.

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The purified recombinant Zika virus of the invention comprises antigens of Zika virus comprising the envelope (E) protein, membrane (M) protein and optionally the non-structural 1 (NS1) protein as vaccine antigens for eliciting immune response for prophylaxis of Zika virus infections, wherein the Zika virus has the structural protein sequences as disclosed in SEQ. ID No. 3 and SEQ. ID No.4 corresponding to nucleotide sequences of SEQ ID. No. 1 and SEQ ID No.2 respectively, for use as vaccine antigens against Zika virus infections caused by genotypes or variants thereof. The Recombinant DNA constructs comprises a (i) vector (ii) at least one nucleic acid fragment corresponding to SEQ ID NO.1 or SEQ ID NO. 2 encoding the amino acid sequence of the proteins of SEQ ID NO.3, SEQ ID NO.4 respectively which is applicable to any Zika virus protein sequences that share at least 70% amino acid identity to the aforementioned SEQ ID NO. 3 and SEQ ID NO.4. The composition of the invention comprises recombinant DNA construct, wherein the vector is an eukaryotic plasmid vector being cloned in a eukaryotic host such as baculovirus for expression in insect cells as virus like particles (VLPs).

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The recombinant protein of Zika virus is obtained by the process comprising the steps of:

- a. transfecting the recombinant plasmid DNA in insect cells;
- b. harvesting the cells and isolating the recombinant protein therefrom;
- c. purifying the protein by a method selected from ion exchange chromatography, gel filtration, affinity chromatography, hydrophobic column chromatography, mixed mode resin chromatography, diafiltration, ultracentrifugation, density gradient centrifugation and fractionation with salt.

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The structural antigens of Zika virus are expressed in any prokaryotic or eukaryotic expression system including baculovirus mediated expression in insect cells.

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The vaccine composition of the invention is obtained by a process wherein neutralizing antibodies are largely elicited against the Envelope protein such as in optimally inactivated virus, live attenuated virus, deactivated virus, DNA vaccine, virus like particles, chimeric virus particles that display the Zika virus E protein in any heterologous virus backbone such as in vectored vaccines and synthetic virus particles derived from any Zika virus genomic RNA sequence.

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The vaccine composition of the invention may further comprise an adjuvant, wherein the adjuvant is selected from the group consisting of a) aluminum salts comprising aluminum hydroxide, aluminum phosphate, aluminum sulphate phosphate; b) inulin; c) algammulin which is a combination of inulin and aluminium hydroxide; d) monophosphoryl lipid A (MPL); e) resiquimod; f) muramyl dipeptide (MDP); g) N-glycolyl dipeptide (GM DP); h) polyIC; i) CpG oligonucleotide; j) aluminum hydroxide with MPL; k) any water in oil

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emulsion; l) any oil in water emulsion that contains one or more of the following constituents: squalene or its analogues or any pharmaceutically acceptable oil, tween-80, sorbitantriolate, alpha-tocopherol, cholecalciferol and aqueous buffer, or any of the analogues and derivatives of the molecules thereof i) two or more combination of any of the
5 aforementioned adjuvants when formulated with Zika virus antigens elicits immune response against the virus. In one preferred embodiment the composition comprises aluminum hydroxide in a concentration range of 0.1 mg to 1.5 mg of aluminum per vaccine dose, preferably 0.25 mg to 0.5 mg aluminum per vaccine dose.

10 The adjuvant of the composition of the invention confers mucosal immunity and systemic immunity when administered in mammals.

The vaccine composition with Zika virus antigen is administered at any dose ranging from 0.125 µg to 100 µg per dose with or without an adjuvant, either as a single dose or in two or
15 more doses to elicit an immune response in a mammal.

In one embodiment the invention provides a method of eliciting a protective immune response in mammals including humans comprising administering the vaccine composition of claim 1 by any route comprising intramuscular, intradermal, subcutaneous, intravenous, oral, intranasal or transcutaneous routes.
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The composition of the invention may be administered by any method comprising needles and syringes including pre-filled syringes, microneedle patch, needle-free patch, inhalation and nasal sprays.
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The invention also provides a method of *in vitro* or *in vivo* use of the Zika virus antibodies of the composition for preparation of immunodiagnostic and immunotherapeutic agents for Zika virus infections.

30 In one embodiment the vaccine composition comprises Zika virus and Japanese encephalitis virus antigens in a combination vaccine that elicits protective immune response in mammals against each of the viruses, wherein the Zika virus antigen and Japanese encephalitis virus inactivated antigens are present in the combination vaccine at concentrations ranging 5 µg to 50 µg of each antigen in a pharmaceutically acceptable formulation without an adjuvant, or
35 with an adjuvant.

The adjuvant may be selected from the group consisting of a) aluminum salts comprising aluminum hydroxide, aluminum phosphate, aluminum sulphate phosphate; b) inulin; c) algamulin which is a combination of inulin and aluminium hydroxide; d) monophosphoryl lipid A (MPL); e) resiquimod; f) muramyl dipeptide (MDP); g) N-glycolyl dipeptide (GMDP); h) polyIC; i) CpG oligonucleotide; j) aluminum hydroxide with MPL; k) any water in oil emulsion; l) any oil in water emulsion that contains one or more of the following constituents: squalene or its analogues or any pharmaceutically acceptable oil, tween-80, sorbitantriolate, alpha-tocopherol, cholecalciferol and aqueous buffer, or any of the analogues and derivatives of the molecules thereof i) two or more combination of any of the
40 aforementioned adjuvants when formulated with Zika virus antigens elicits immune response against the virus. In one preferred embodiment, the adjuvant is aluminium hydroxide with 0.25 mg to 1.0 mg of aluminium content per vaccine dose.
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In another embodiment, the vaccine composition comprises Zika virus and Chikungunya virus antigens in a combination vaccine that elicits protective immune response in mammals against each of the viruses, wherein Zika and Chikungunya virus antigens are present in a combination vaccine at concentrations ranging from 5 µg to 50 µg of each antigen in a pharmaceutically acceptable formulation without an adjuvant, or with an adjuvant.

The adjuvant may be selected from the group consisting of a) aluminum salts comprising aluminum hydroxide, aluminum phosphate, aluminum sulphate phosphate; b) inulin; c) alammulin which is a combination of inulin and aluminium hydroxide; d) monophosphoryl lipid A (MPL); e) resiquimod; f) muramyl dipeptide (MDP); g) N-glycolyl dipeptide (GMDP); h) polyIC; i) CpG oligonucleotide; j) aluminum hydroxide with MPL; k) any water in oil emulsion; l) any oil in water emulsion that contains one or more of the following constituents: squalene or its analogues or any pharmaceutically acceptable oil, tween-80, sorbitantriolate, alpha-tocopherol, cholecalciferol and aqueous buffer, or any of the analogues and derivatives of the molecules thereof i) two or more combination of any of the aforementioned adjuvants when formulated with Zika virus antigens elicits immune response against the virus. In one preferred embodiment, the adjuvant is aluminium hydroxide at 0.25 mg to 1.5 mg of aluminium content per vaccine dose.

In another embodiment, the vaccine composition comprises Zika virus, Chikungunya virus and Japanese encephalitis virus antigens in a combination vaccine that elicits protective immune response in mammals against each of the viruses, wherein Zika virus, Chikungunya virus and Japanese encephalitis virus antigens are present in a combination vaccine at concentrations ranging from 5 µg to 50 µg of each antigen in a pharmaceutically acceptable formulation without an adjuvant, or with an adjuvant.

The adjuvant may be selected from the group consisting of a) aluminum salts comprising aluminum hydroxide, aluminum phosphate, aluminum sulphate phosphate; b) inulin; c) alammulin which is a combination of inulin and aluminium hydroxide; d) monophosphoryl lipid A (MPL); e) resiquimod; f) muramyl dipeptide (MDP); g) N-glycolyl dipeptide (GMDP); h) polyIC; i) CpG oligonucleotide; j) aluminum hydroxide with MPL; k) any water in oil emulsion; l) any oil in water emulsion that contains one or more of the following constituents: squalene or its analogues or any pharmaceutically acceptable oil, tween-80, sorbitantriolate, alpha-tocopherol, cholecalciferol and aqueous buffer, or any of the analogues and derivatives of the molecules thereof i) two or more combination of any of the aforementioned adjuvants when formulated with Zika virus antigens elicits immune response against the virus. Preferably, the adjuvant is aluminium hydroxide at 0.25 mg to 1.0 mg of aluminium content per vaccine dose.

The vaccine composition of the invention optionally comprises 2-phenoxyethanol preservative at a concentration of 2.5 to 5 mg/mL.

The vaccine composition when administered in a single dose or in two or more doses in mammals elicits both Th1 and Th2 immune response against any of the arbovirus antigens comprising Zika Virus, Chikungunya virus and Japanese Encephalitis virus and is suitable for administration to humans.

In one embodiment the invention provides a method for preparation of a vaccine composition comprising one or more arbovirus antigens selected from Zika virus, Chikungunya virus and Japanese encephalitis virus, the method comprising one or more steps of inactivation,

producing recombinant protein, expressing structural antigens, purification and concentration of the virus antigen wherein said purification and concentration of Zika virus comprises one or more steps selected from:

- 5 a. ultracentrifugation ;
- b. density gradient centrifugation;
- c. clarification of the viral harvest using membrane filtration;
- d. purification by column chromatography;
- 10 e. tangential flow filtration using membranes with cut off from 100 kDa to 300 kDa, wherein tangential filtration is carried out either before or after virus inactivation.

The column chromatography method comprises gel filtration, mixed mode resin column chromatography, any ion exchange column chromatography, affinity matrix chromatography and hydrophobic interaction chromatography, wherein the column chromatographic method 15 elutes majority of the virus antigen in the flow through such as Capto Core 700, most preferably Capto Core 700 wherein the virus sample is purified on Capto Core 700 column and is eluted in the flow through.

The Zika virus is inactivated by one or more inactivating agents selected from a chemical 20 inactivating agent, a physical inactivating agent and an irradiating agent.

The preparation method comprises inactivation of Zika virus which may be carried out before or after purification of the virus, wherein the Zika virus may be inactivated by chemical 25 inactivating agent selected from formalin (formaldehyde), beta propiolactone (BPL) and hydrogen peroxide.

In one embodiment, the preparation method comprises inactivation of the Zika virus bulk which is inactivated by any one of the following methods selected from:

- 30 a. Formalin treatment at any concentration ranging from 1: 500 up to 1: 4000 v/v of formalin: virus, at 8⁰C to 37⁰C, preferably 25±3⁰C, for at least 1 to 7 days;
- b. Formalin treatment at any concentration ranging from 1:500 up to 1: 4000 v/v of formalin: virus, at 2⁰C to 8⁰C for at least 10 to 30 days;
- 35 c. Beta propiolactone (henceforth BPL) at any concentration ranging from 1:500 up to 1: 4000 v/v of BPL: virus, for at least 24 to 48 hrs, if not more, at temperatures ranging from 8⁰C to 30⁰C, preferably 25±3⁰C, for 48 hours;
- d. Beta propiolactone at any concentration ranging from 1: 500 up to 1:4000 (BPL: virus, v/v), at 2⁰C to 8⁰C for at least 3-7 days;
- 40 e. a combination of BPL and formalin at any of the aforementioned conditions, preferably BPL inactivation at 1:3000 (BPL :virus, v/v) for 24 hours followed by formalin inactivation at 1: 3000 (formalin: virus, v/v) for 24to 48 hours at 15⁰C to 30⁰C, preferably 25±3⁰C ;
- f. hydrogen peroxide at any concentration from 0.1 to 3%, preferably 0.1 to 1% at any temperature from 20 - 30⁰C for 5 minutes to 120 minutes.

45 In embodiment of preparation method, the virus is inactivated by gamma irradiation by exposure from 20 kGy (Kilo Gray) up to 35 kGy, preferably 25 kGy to 30 kGy from a ⁶⁰Co source.

50 In another embodiment of preparation method, the Zika virus is inactivated by UV irradiation by exposure to 254 nm for 30 – 60 minutes.

In another embodiment of the preparatory method, the Zika virus is inactivated by heat treatment from 50°C to 65°C for 30 min up to 2 hrs, preferably, 65°C for 1 hr.

In one embodiment of the preparation method, the inactivation is carried out in the presence of stabilizing agent selected from lactose, sucrose, trehalose, maltose, mannose, iso-maltose, raffinose, stachyose, lactobiose, sorbitol, mannitol, lactobionic acid, dextran, L-glycine, L-histidine, L-glutamic acid, L-aspartic acid and human serum albumin or combinations thereof. In one preferred embodiment the stabilizing agent is selected from:

- a. 2% sorbitol and 1% L-glycine;
- b. 1% sorbitol and 0.5 % L-glycine;
- c. 1% mannitol and 0.5% L-glycine;
- d. 1% mannitol and 0.5% L-glutamic acid; and
- e. 1% sorbitol and 0.5% L-glycine, 1% human serum albumin.

The inactivation methods described hereinabove are applicable to Zika virus of any genotype/strain, live attenuated Zika virus, deactivated virus, virus like particles, chimeric virus particles that carry any Zika virus antigens particularly the E protein in any heterologous virus backbone, in vectored vaccines and infectious synthetic virus particles derived *in vitro* or *in vivo* using the sequence of any Zika virus genome.

In one embodiment the invention discloses a method of producing the recombinant protein comprising the steps of:

- a. transfecting recombinant plasmid DNA in insect cells;
- b. harvesting the cells and isolating the recombinant protein therefrom;
- c. purifying the protein by at least one of the methods comprising of ion exchange chromatography, gel filtration, affinity chromatography, hydrophobic column chromatography, mixed mode resin chromatography, diafiltration, ultracentrifugation, density gradient centrifugation, fractionation with salt.

In another embodiment, the invention discloses method of expressing the structural antigens of Zika virus comprising expression system is any prokaryotic or eukaryotic expression system including baculovirus mediated expression in insect cells.

In another embodiment the invention discloses a method wherein the method comprises neutralizing antibodies that are largely elicited against the Envelope protein such as in optimally inactivated virus, live attenuated virus, deactivated virus, DNA vaccine, virus like particles, chimeric virus particles that display the Zika virus E protein in any heterologous virus backbone such as in vectored vaccines and synthetic virus particles derived from any Zika virus genomic RNA sequence.

The vaccine composition of the invention may be administered in a prime boost strategy, wherein the prime is the candidate inactivated vaccine and the boost is either the same vaccine or any other vaccine such as DNA vaccine, Chimeric Zika virus vaccine, virus like particles, deactivated Zika vaccine, live attenuated virus vaccine, recombinant subunit vaccine, vectored vaccine or any vaccine derived from synthetic Zika virus, wherein the neutralizing antibodies in each of them are elicited against Zika virus Envelope protein.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Fig. Purified Zika virus bulk in 12.5% SDS-PAGE gel detected by silver staining. The Envelope (E) protein and the Membrane (M) proteins are the major proteins detected in the purified antigen

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Figure 2: Fig.2A – Inactivation kinetics of Zika virus by formalin at concentrations ranging from 1:1000 v/v of formalin: virus up to 1:4000 v/v of formalin: virus at $25\pm 3^{\circ}\text{C}$. Fig.2B – inactivation kinetics of Zika virus by beta-propiolactone at concentrations ranging from 1:1000 up to 1:3500 v/v of BPL: virus at $25\pm 3^{\circ}\text{C}$. In both the inactivation procedures, 1% sorbitol and 0.5% L-glycine (final concentration) were added as stabilizers, which had no effect on the inactivation kinetics. The inactivated samples were serially amplified three times *in vitro* in Vero cells, and assayed at the end of three passages by TCID₅₀.

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Figure 3: Fig.3A: The ~2.1 kb Zika virus prME gene of SEQ ID NO.1 was amplified by gene specific primers for initiating cloning in pFastBac vector for expression in insect cells. Fig.3B: The Sf9 cell lysate was probed by Western for detection of expression of the prME protein using Zika rabbit polyclonal antisera by standard procedures. The envelope protein of ~ 55 kD could be detected as the major band.

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Figure 4: Estimation of neutralizing antibody titers elicited by Zika vaccine formulations with different adjuvants. Adjuvants are abbreviated as follows: pIC (polyIC); C- cholecalciferol; MPL (lipid A; monophosphoryl); RP (resiquimoid + polyIC); RM (resiquimoid + OWEM2); I (inulin); OWEM2 (oil in water emulsion 2); AI (aluminum hydroxide + inulin); MDP (muramyl dipeptide); OWEM1 (oil in water emulsion 1). No significant antibody titers could be detected in the respective control groups and hence not depicted in the figure. In all cases, 10 µg of two doses of Zika vaccine antigen was formulated for administration in Balb/c mice by IM route.

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Figure 5: Fig. 5A: Estimation of neutralizing antibody titers by PRNT₅₀ in dose ranging studies from 0.125 µg up to 40 µg per dose of the aluminum hydroxide adjuvanted formalin inactivated Zika virus vaccine administered by IM route in Balb/c mice in two doses. Fig.5B: the vaccinated animals were challenged intravenously with 10e5 PFU /animal of the Zika virus strain 7 days after the booster dose, and viremia was monitored every 24 hours for 7 days (depicted in the graph for 6 days). All the animals showed complete protection from viremia whereas the animals administered the placebo control showed viremia that persisted up to 6 days. The infectious virus was estimated in the blood samples by TCID₅₀.

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Figure 6: Virus challenge in 4-6 week old Balb/c mice after administration of 1 µg to 40 µg of BPL inactivated, alum adsorbed Zika virus vaccine. Animals of all the vaccine dose groups and the placebo group were challenged with 10e5 PFU of Zika virus MR766 strain 7 days after administration of the booster dose. Viremia was monitored every 24 hours after virus challenge, and the titers of infectious particles in blood were estimated by TCID₅₀. The candidate Zika vaccine offered complete protection from virus challenge at all dose groups.

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Figure 7: Passive immunization offered complete protection against viremia and infectious virus could not be detected by TCID₅₀ in the animals that received Zika rabbit polyclonal antisera intraperitoneally and challenged 24 later with 10e5 PFU of Zika virus. Infectious virus particles could not be detected by TCID₅₀ in the blood, when monitored every 24 hours for 6 days, whereas the control animals that received equal volume of PBS showed persistent viremia up to 6 days when challenged with the same dose of the virus.

Figure 8: Formalin inactivated, alum adsorbed Zika virus vaccine antisera from vaccinated mice neutralized the homologous MR766Zika virus strain (Fig.8B) and cross neutralized the heterologous Asian genotype FSS 13025strain (Fig.8A) with equal efficiency with PRNT₅₀ titers of 18105 and 18325 respectively. The values for the placebo (alum only) are also depicted in the graph alongside.

Figure 9:Antibody titers expressed as log₁₀ of reciprocal of serum dilutions from the dose ranging studies with (Fig.9A) single dose (Fig.9B) two doses (Fig.9C) three doses of formalin inactivated vaccine administered in 4-6 week old Balb/c mice as described in Example 7. Fig.9D is antibody titers with a single, two and three doses of 10 µg of vaccine antigen without alum. All values are expressed as Geometric Mean Titers with 95% CI. In 9A-9D, individual animal data is plotted. Zika virus antigen was immunogenic even without an adjuvant. Data from other dose ranges were estimated but not included in the graph.

Figure 10: High affinity antibodies could be elicited by single dose of formalin inactivated alum adsorbed Zika virus vaccine in Balb/c mice even at low doses of the vaccine antigen up to 1 µg. Antibody avidity was expressed as avidity index and estimated by methods described in Example 12.

Figure 11: Estimation of Th1 cytokines(Fig.11A) IFN gamma, and (Fig.11B) IL-2 in mice vaccinated with Zika vaccine formulations with different adjuvants. In all cases it was 10 µg of vaccine antigen per dose. Adjuvants are abbreviated as follows: pIC (polyIC); C-cholecalciferol; MPL (lipid A; monophosphoryl); RP (resiquimoid + polyIC); RM (resiquimoid + OWEM2); I (inulin); OWEM2 (oil in water emulsion 2); AI (aluminum hydroxide + inulin); MDP (muramyl di peptide); OWEM1 (oil in water emulsion 1) as described in Example 5. Oil based adjuvants and polyIC elicited a strong Th1 response compared to other adjuvants tested.

Figure 12:Estimation of Th2 cytokines (Fig.12A)IL-4, and (Fig.12B) IL-10, in mice vaccinated with Zika vaccine formulations with different adjuvants. In all cases it was 10 µg of vaccine antigen per dose. Adjuvants are abbreviated as follows: pIC (polyIC); C-cholecalciferol; MPL (lipid A; monophosphoryl); RP (resiquimoid + polyIC); RM (resiquimoid + OWEM2); I (inulin); OWEM2 (oil in water emulsion 2); AI (aluminum hydroxide + inulin); MDP (muramyl di peptide); OWEM1 (oil in water emulsion 1) as described in Example 5. Oil based adjuvants and polyIC elicited strong Th2 response in addition to Th1 response.

DETAILED DESCRIPTION OF THE INVENTION

This disclosure concerns formulation of immunogenic compositions. The invention discloses in particular, preparation and formulation of vaccine antigens of Zika virus in monovalent compositions and in combination with other arboviruses such as Chikungunya and /or Japanese encephalitis viruses. In particular, the invention discloses compositions for prophylaxis and treatment of Zika virus infections.

One aspect of the invention is that the methods of preparation, formulation and use of Zika antigens as vaccine for eliciting immune response is applicable to any genotype, genotypic variants or any strain of Zika virus wherein one genotype of Zika virus cross neutralizes a heterologous strain efficiently. The Zika virus can be selected from Asian, West African or East African genotype of the virus. Therefore, the methods described in the current invention herein are applicable to Zika virus of any genotype/strain, live attenuated Zika virus, deactivated virus, virus like particles, chimeric virus particles that carry any Zika virus antigens particularly the E protein and the M protein in any heterologous virus backbone, in vectored vaccines and infectious synthetic virus particles derived *in vitro* or *in vivo* using the sequence of any Zika virus genome. A chimeric virus has the nucleic acid of a heterologous virus and nucleic acid of Zika virus.

In the context of the immunogenic compositions disclosed herein, in particular the bulk antigen used for preparation of immunogenic compositions, the methods of preparation, formulations and use of Zika vaccine antigens are applicable to any of the aforementioned Zika virus types, that share at least 50% amino acid identity and up to 100% amino acid identity across any region of the genome. In the context of the immunogenic compositions disclosed herein, sequence of Zika virus MR766 strain of African genotype (SEQ ID NO.5 for genomic nucleotide sequence and SEQ ID NO.6 for complete ORF) shares more than 96.5% amino acid identity in the structural Envelope protein with the Asian genotype strain FSS13025 and whose sequence is disclosed in SEQ ID NO.7 and SEQ ID NO.8 for the nucleotide and protein sequences respectively. Vaccine antisera of the MR766 strain cross neutralized the FSS13025 strain with 100% equivalent potency as the homotypic MR766 strain. Also in the context of the disclosure herein, Zika virus prME (SEQ ID NO.3) antisera efficiently cross neutralized the MR766 strain confirming that all Zika viruses are serotypically similar. In the context of the disclosure herein, the vaccine methods developed using any one of the Zika virus strains is applicable to homologous and any heterologous Zika virus strains for use as candidate vaccine.

A cell line that can be propagated *in vitro* in culture can be used as a host for Zika virus culture. For propagating Zika virus strains, preferably permissive cells which allow the virus to grow well are selected. For example, diploid cell lines such as MRC-5 and WI-38, and serially passaged cell lines such as Vero, BHK-21, CHO cells etc. can be used. For example, Vero cells (ATCC No. CCL-81), BHK-21 (ATCC No. CCL-10), C6/C3 (ATCC No. CRL-1660) etc. can be used. In a preferred embodiment, one such cell line used in the current invention is Vero cells (ATCC No. CCL-81) which has been validated for use as a host cell for vaccine production. The validated Vero cell lines conforms to the Requirements for Biological Substances No.50 regarding requirements for use of cells for the production of biologicals recommended by the World Health Organization (WHO) thereby confirming these cell lines as qualified for producing a vaccine (WHO Technical report Series, No. 878, pp 19-52, 1998).

In one aspect of the invention, the method of adaptation of Zika virus to Vero cells increases the virus titer. Zika virus passaged repeatedly in Vero cells increases the virus titer. In the context of virus growth in Vero cells disclosed herein, Zika virus passaged initially in mouse brain or *Ae.albopictus* C6/36 cells (ATCC No. CRL-160) and then adapted to Vero cells increases virus titers suitable for vaccine production.

For maintenance in cell culture of the above-mentioned cell lines, Vero cells in particular, stationary culture in monolayers, perfusion system culture, shake flasks, roller tube/bottle culture, suspension culture with and without microcarriers, cell factories and cell stacks, bioreactors and disposable bioreactors, wave bioreactors and the like can be adopted. For example various types of microcarriers are commercially available. Commercially available animal cell culture devices can be used to facilitate the growth of cells to high cell density.

In one aspect of the invention and disclosed herein, the Zika virus is purified for use as candidate vaccine. Purification is achieved by a combination of both physical and chemical methods either before or after inactivation of the virus. Physical methods include any of the following techniques but not limited to: ultracentrifugation, density gradient centrifugation, ultrafiltration, diafiltration and concentration using semi-permeable membranes with suitable molecular cut-off sizes. Purification through chemical means employs methods such as adsorption/desorption through chemical or physiochemical reactions such as ion exchange chromatography, affinity chromatography, hydrophobic interaction chromatography, gel filtration chromatography such as for example Captocore700TM, hydroxyapatite matrix, salting with inorganic salts, one such example being ammonium sulphate.

In a preferred embodiment, the virus is purified on Capto core 700 (GE Healthcare Life Sciences) column chromatography. Inactivation of the virus is achieved either before purification or after purification on Capto core 700 column. The virus harvest before Capto core 700 column can be clarified using membrane filters with different pore sizes, preferably not less than 0.45 μM low protein binding membrane. In a preferred embodiment, the virus harvest can be clarified with a dual membrane of two different pore sizes, for example 1.2 μM followed by 0.45 μM , or 0.8 μM followed by 0.45 μM . The clarified virus harvest is suitable for purification on Capto Core 700 column. The buffers used for purification on Capto core 700 is of optimal pH and ionic strength to maximize the binding of the impurities on the column and elute the virus in the flow through. The virus sample is further concentrated by diafiltration before or after virus inactivation. Diafiltration of the virus sample after inactivation removes the virus inactivating agent from the bulk antigen, and is suitable for formulation.

In one embodiment of the invention, Zika virus is inactivated (killed) for use as a vaccine antigen. Inactivation can be carried out either before or after purification of the virus. In a preferred embodiment, inactivation of Zika virus is carried out after purification of the virus.

Zika virus can be inactivated either by heat, gamma irradiation, ultraviolet light or by chemical means. In a preferred embodiment disclosed herein, Zika virus is chemically inactivated. Chemical inactivating agents were selected from the following list which includes but is not limited to: formalin, beta-propiolactone, glutaraldehyde, N-acetyleneimine, binary ethyleneimine, tertiary ethyleneimine, ascorbic acid, caprylic acid, psolarens, detergents including non-ionic detergents etc. wherein the chemical inactivating agent is added to a virus suspension to inactivate the virus.

In a preferred embodiment of the invention, the chemical inactivating agent selected is formalin and/or beta propiolactone (BPL). Formalin is used at any concentration ranging from 1:1000 to 1:4000 v/v of formalin: virus. Beta propiolactone is used at any concentration ranging from 1:1000 to 1:4000 v/v of BPL: virus. The temperature and duration of inactivation is optimized to complete virus inactivation with minimal adverse effect on immunogenicity. This can be achieved with shorter duration of exposure with minimum quantity of the inactivating agent. In the context of virus inactivation, the disclosure herein describes the concentration, temperature and time of exposure of Zika virus to formalin and BPL. In the preferred embodiment of the invention, the inactivation temperature is $25\pm 3^{\circ}\text{C}$, most preferably 22°C for 7 days. At lower temperatures of 2°C to 8°C , the duration of formalin exposure is longer than 7 days to achieve complete virus inactivation at the aforementioned concentration ranges. Duration of virus exposure to formalin can be reduced to below 48 hours by increasing the temperature of exposure up to 37°C . Hence, effective formalin inactivation of Zika virus can be achieved at any concentration range of formalin from 1:1000 v/v formalin: virus up to 1:4000 v/v formalin: virus by choosing any temperature range from 2°C to 37°C and varying the exposure time from 24 hours to more than 10 days at any of the aforementioned concentrations, time and temperature of exposure.

In one embodiment of the disclosure, BPL is used as virus inactivating agent for Zika virus. In a preferred embodiment of the invention, BPL is used at concentrations ranging from 1:1000 v/v BPL: virus up to 1: 4000 v/v BPL: virus. At lower temperatures of 2 to 8°C , the duration of BPL exposure is preferred for 3 to 7 days to achieve complete virus inactivation at the aforementioned concentration ranges. Duration of virus exposure to BPL can be reduced to 48 hours or below by increasing the temperature of exposure up to $25\pm 3^{\circ}\text{C}$ or even up to 37°C . Hence, effective BPL inactivation of Zika virus can be achieved by choosing any concentration range of BPL from 1:1000 v/v BPL: virus to 1:4000 v/v BPL: virus by choosing any temperature range from 2 to 37°C and varying the exposure time from 24 hours to more than 10 days at any of the aforementioned concentrations, time and temperature of exposure.

One of embodiments of the current invention disclosed herein is the use of a combination of BPL and formalin at any of the aforementioned conditions, preferably BPL inactivation at 1:3000 v/v of BPL: virus for 24 hours followed by formalin inactivation at 1: 3000 v/v formalin: virus for 24to 48 hours at 15°C to 30°C , preferably $25\pm 3^{\circ}\text{C}$. The use of BPL and formalin combination for Zika virus inactivation is that the mechanism of inactivation being different for formalin and BPL, their combined used reduces their overall concentration and exposure to both the inactivating agents, and also the use of low concentrations of formalin promotes stability of the virus bulk by promoting cross linking of virus epitopes. In another embodiment of the invention, hydrogen peroxide is used for inactivating the Zika virus at concentrations ranging from 0.1 to 3%, preferably 0.1 to 1% at any temperature from 20°C to 30°C for 5 to 120 minutes, if not more.

An embodiment of the current invention discloses the use of the prME antigen of Zika virus as the candidate vaccine antigen to elicit immune response against the Zika virus. The disclosure is applicable to any method of vaccine design wherein the prME or the E protein is expressed in such a manner that the neutralizing Zika antibodies are directed against the said antigens. In a preferred embodiment of the invention, the prME protein is expressed as recombinant virus like particles (VLP) in baculovirus mediated expression in insect cells. Anyone skilled in the art will derive additional embodiments using the above disclosure to design a vaccine candidate using the prME protein as the target Zika antigen such as a DNA

vaccine, Virus like particles comprising prME proteins, subunit vaccine comprising the Envelope (E) antigen, live vectored vaccines, chimeric vaccines using the Zika prME on a heterologous nucleic acid backbone wherein in all the above the anti-Zika antibodies are directly against the E protein.

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In the current invention disclosed herein, are immunogenic compositions comprising purified recombinant Zika virus antigens comprising the envelope (E) protein, membrane (M) protein and optionally the non-structural 1 (NS1) protein as vaccine antigens for eliciting immune response for prophylaxis of Zika virus infections. In a preferred embodiment, the use of the Zika virus having of the prME gene of sequences SEQ ID NO.1 and SEQ ID NO.2 encoding the structural protein of SEQ. ID NO. 3 and SEQ. ID NO.4 respectively, wherein the expressed and purified prME protein can be used as vaccine antigen for prophylaxis of Zika virus infections.

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In a preferred embodiment, Zika virus prME gene is used to generate a recombinant gene construct that can be used to express the prME protein in prokaryotic or eukaryotic expression systems as virus like particles (VLPs), preferably baculovirus mediated expression in insect cells. The methods disclosed herein are applicable to any Zika virus strain that share at least 70% amino acid identity to the aforementioned SEQ ID NO. 3 and SEQ ID NO.4

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An embodiment of the current disclosure is the choice of pharmaceutically acceptable buffer throughout the bioprocess wherein the buffering agent is selected from a list consisting of any one or more of the following, but not limited to: phosphate buffer; citrate buffer; phosphate citrate buffer; borate buffer; tris(hydroxymethyl)aminomethane (Tris) containing buffer; succinate buffer; buffers containing glycine or histidine as one of the buffering agents. In the most preferred embodiment, phosphate buffer is used, wherein phosphate buffer is sodium phosphate buffer at concentration of 5 mM up to 200 mM of phosphate ions, preferably 10 mM to 100 mM phosphate buffer, most preferably 10 mM to 50 mM phosphate buffer of any pH above 6.50 to pH 9, preferably pH 6.8 to pH 7.8 is used for the upstream and downstream processes. In a preferred embodiment, 10 mM sodium phosphate buffer of pH 7.4±0.2 is used in the preparation of the purified inactivated vaccine bulk of Zika virus antigen, and optionally containing sodium chloride at a concentration from 50 to 200 mM. In another preferred embodiment, sorbitol and L-glycine are optionally added to a final concentration of 1% and 0.5% respectively.

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An embodiment of the current invention also discloses the choice of adjuvants that is compatible for formulation with Zika virus antigen.

The antigenic compositions of Zika virus as monovalent vaccine, and with Chikungunya virus and Japanese encephalitis viruses in combination vaccine were formulated in pharmaceutically acceptable carrier for immunization. The use of adjuvant(s) can reduce the amount of antigen required in the formulation Furthermore, for adjuvanted vaccine formulations, suitable adjuvant(s) were selected from the following list, which includes but is not limited to: alum such as aluminum hydroxide, aluminum phosphate, or amorphous aluminum sulphate phosphate; calcium phosphate; inulin of any polymorphic form, preferably gamma inulin; adjuvants containing inulin in combination with other organic and inorganic compounds such as aluminum hydroxide, aluminum phosphate, aluminum sulphate phosphate and calcium phosphate; liposomes, chitosan and complex carbohydrates such as dextran, dextrans, starch, mannans and glucomannans, galactomannans, beta-glucans, heparin, cellulose, hemicellulose, pectins and pectinates, lectins and any other carbohydrates

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either synthetic or derived from any source, any biodegradable and biocompatible polymers, such as poly lactide and polylactide co-glycolides, (PLG or PLGA); any emulsions including but not limited to oil in water emulsions one such example being squalene or squalene analogues containing oil in water adjuvants, oil in water emulsions containing vegetable oils; 5 any water in oil emulsion; liposomes prepared with cholecalciferol as one of the ingredients along with other lipid soluble compounds; liposomes of other compositions; RIBI adjuvant systems, saponins including but not limited to QS-21, QuilA, tomatine, ISCOMs, ISCOMATRIX etc, lipopeptides, glycopeptides and their analogues, resiquimoid, lipopolysaccharides, lipid A, muramyl dipeptides or their analogues and any peptide based 10 adjuvants, oligonucleotides, any TLR ligands and their analogues as adjuvants, any cytokine, vitamins and non-toxic bacterial toxins, indeed any analogues of all the aforementioned adjuvants and combination of two or more of the aforementioned adjuvants or their analogues that are compatible in vaccine formulation(s). and tested for enhanced immunogenicity. In addition to the above, any other organic and inorganic substances that 15 have good immunopotentiating activity are suitable to be used as adjuvant either singly or in adjuvant combinations to enhance the immunogenicity of the arboviral antigens. The use of adjuvant in the vaccine formulations can reduce the amount of antigen required.

In a preferred embodiment of the invention, aluminum hydroxide was used for dose ranging 20 studies of both formalin and BPL inactivated Zika antigens as well in vaccine combinations of Zika, CHIKV and JEV vaccines due its safety profile for use in target population. Oil based emulsions and polyIC gave good immunopotentiating effect to Zika antigen when used as adjuvants. In one embodiment of invention, polyIC and other adjuvants that offer both 25 systemic mucosal immunity is particularly advantageous for protection against disease caused by Zika virus infections. PolyIC and the oil based emulsions and the adjuvant combinations disclosed in the invention elicited both Th1 and Th2 responses estimated by the measurement of the Th1 and Th2 cytokines after vaccination.

In one embodiment of the current invention, a vaccine preservative is used in the vaccine 30 formulations. The preferred embodiment is 2-phenoxy ethanol at a concentration of 2.5 to 5 mg per dose.

In one aspect of the current invention disclosed herein are the use of stabilizing agents selected from one or more of the following, but not limited to: lactose, sucrose, trehalose, 35 maltose, mannose, iso-maltose, raffinose, stachyose, lactobiose, sorbitol, mannitol, lactobionic acid, dextran, L-glycine, L-histidine, L-glutamic acid, L-aspartic acid, human serum albumin and combinations thereof, at any suitable concentration that are used to confer stability during the inactivation of Zika virus by any of the aforementioned methods. In a preferred embodiment, the stabilizing agents are selected from any of the following 40 combinations but not limited to: 2% sorbitol and 1% L-glycine; 1% sorbitol and 0.5 % L-glycine; 1% mannitol and 0.5% L-glycine; 1% mannitol and 0.5% L-glutamic acid; 1% sorbitol, 0.5% L-glycine, 1% human serum albumin. In a preferred embodiment, the combination of 1% sorbitol and 0.5% L-glycine and 1% mannitol and 0.5% L-glycine are preferred combinations, most preferably, 1% sorbitol and 0.5% L-glycine. One skilled in the 45 art will recognize further embodiments based on the above disclosures.

Lyophilized formulations are one of the methods for preparation of vaccine product. Lyophilized preparations of Zika virus vaccine typically contain purified inactivated Zika 50 virus, a sugar polyol, preferably sorbitol and mannitol, most preferably sorbitol in

combination with a glass forming sugar, which is preferably a disaccharide or an oligosaccharide. The preferred disaccharide is selected from the following list but is not limited to: sucrose, trehalose, maltose, mannose, lactose, raffinose, isomaltose, stachyose etc. the preferred embodiment of the disclosure is a combination of 1% sorbitol with 5% sucrose, 1% mannitol with 5% sucrose, and 3% sucrose and 2% trehalose, 1% mannitol with 1% L-glycine and or 2% trehalose. Any one of the ordinary skill in the art will devise further embodiments and based on the disclosures above.

The lyophilized formulations can be re-suspended in water for injection or an aqueous buffer that is pharmaceutically acceptable for administration. e.g. as an injectable liquid to a human subject. The lyophilized formulation can also be used as an inhalable powder which will be suitable for inducing mucosal immunity. Additionally the lyophilized formulation of Zika virus can comprise an adjuvant that confers mucosal immunity preferably from a list of those adjuvants tested in the current invention for Zika virus such as polyIC for example.

In the current invention, the disclosure provided herein on the optimal use of Zika virus antigen to elicit robust immune response, the vaccine antigen can be used at 0.10 μg up to 100 μg per dose, wherein the preferred embodiment is any concentration from 0.125 μg up to 40 μg per dose such that the administered vaccine doses elicit antibody titers measurable by assays such as ELISA and PRNT₅₀. The vaccine can be administered with and without an adjuvant as both the inactivated vaccine and the adjuvanted formulations elicit good immune response.

In yet another disclosure of the invention, the inactivated Zika vaccine candidate inactivated by any of the disclosed methods can be administered as a single dose or in two or more doses to elicit immune response. The methods disclosed in the invention provide the kinetics of immune response after each dose of the vaccine, at dose ranges from 0.125 μg up to 40 μg per dose that offers the flexibility of the choice of the vaccine dose range concentrations and number of doses to suit the target population for vaccination.

The route of vaccine administration can be by any route selected from, but not limited to intramuscular, intradermal, subcutaneous, intravenous, oral, intranasal and transcutaneous routes. In a preferred embodiment of the invention, the preferred route of vaccine administration is intramuscular (IM) route.

The vaccine formulations can be presented in glass vials and injected by needle and syringes, presented in pre-filled syringes in a ready to use presentation or administered by electroporation, microneedle patches, needle free patch, by inhalation or by nasal sprays.

The current invention discloses methods for preparation and use of formulations comprising one or more arbovirus antigens selected from a list that includes Zika virus, Chikungunya virus (CHIKV), and Japanese encephalitis virus (JEV). When used in vaccine combination, the vaccine can elicit immune response against each of the viruses present in a combination vaccine. In a preferred embodiment of the invention comprising a vaccine composition wherein Zika virus antigens and Japanese encephalitis virus antigens are present in a combination vaccine at concentrations ranging from 5 μg to 50 μg of each antigen in a pharmaceutically acceptable formulation without an adjuvant, or preferably with an adjuvant selected from the list of adjuvants disclosed in the current invention, preferably aluminum hydroxide with 0.25 mg to 1.5 mg of aluminum content per vaccine dose is disclosed. In yet another preferred embodiment of the invention a vaccine composition comprising

Chikungunya and Zika virus antigens in a formulation comprising 5 µg to 50 µg of each antigen in a pharmaceutically acceptable formulation without an adjuvant, or preferably with an adjuvant selected from the list of adjuvants disclosed in the current invention, preferably aluminum hydroxide with 0.25 mg to 1.5 mg of aluminum content per vaccine dose is disclosed.

In yet another preferred embodiment of the invention, a vaccine composition comprising Chikungunya, Zika and JEV virus antigens in a formulation comprising 5 µg to 50 µg of each antigen in a pharmaceutically acceptable formulation without an adjuvant, or preferably with an adjuvant selected from the list of adjuvants disclosed in the current invention, preferably aluminum hydroxide with 0.25 mg to 1.5 mg of aluminum content per vaccine dose is disclosed. The use of vaccine combination confers a distinct economical advantage for manufacture and distribution of vaccines, provided that immune response is elicited against each of the antigen in the formulation and no antigenic interference is observed to either of the antigen by the presence of an additional antigen. The vaccine antigens can either be administered from a single formulation or administered separately at the same time or in suitable time intervals so as to elicit an immune response to the cognate antigen.

The current invention also discloses the use of Zika virus antibodies for detection of Zika virus by ELISA or in any immunodiagnostic methods where the antibodies find an application for detection or diagnosis of Zika virus infections.

The current invention also discloses herein the use of Zika virus antibodies for prevention and treatment of Zika virus disease.

Abbreviations used in the invention: IM – intramuscular; mcg-microgram; TCID₅₀- 50% Tissue Culture Infectious Dose; PFU – Plaque forming unit

EXAMPLES

Example 1: Zika Virus culture in Vero cells

Vero cell line (ATCC No. CCL-81) was used as the cell substrate for culture of Zika virus. Extensively characterized Vero cells obtained from BioReliance, USA was used in pilot scale production. Vero cells were grown in DMEM (Dulbecco's Modified Eagle Medium; Sigma-Aldrich Catalog # D5523 and used as per the manufacturer's instructions) or EMEM (Eagles Minimal Essential Medium) containing 5% fetal bovine serum (FBS) or New Born Calf Serum (NBCS) and incubated at 35°C to 37°C until reaching 80 – 100 % confluence of the monolayer. Post-infection, the same medium containing 1% serum was used, or alternatively the virus was cultured in Vero cells adapted to serum free medium. Zika virus also could be grown in MRC-5 cell monolayer which were prepared in growth medium consisting of EMEM buffered to neutral pH with Hepes buffer with 5% serum and statically incubated at 35°C to 37°C for 6 to 8 days. Zika virus was cultured routinely in Vero cells. Zika virus MR766 strain (ATCC VR-84) was adapted to Vero cells by direct inoculation in Vero cells. Alternatively, the virus was adapted in C6/36 *Ae. albopictus* cells twice by serial passages, and the Zika virus in culture supernatant from these cells was used to infect Vero cells. Serial passage of Zika virus in C6/36 cells cultured at 25°C to 28°C increased the virus titer higher than 10e8.0 TCID₅₀/mL or 10e8.0 PFU/mL. This also obviated the need for subsequent repeat passages in Vero cells to obtain high titers. Virus adaptation by this method is useful to achieve high titers and subsequent higher yield in production. After culture in C6/36 cells, the virus was serially plaque purified twice from Vero cells, and the virus from a single well

isolated plaque was amplified and extensively characterized to be free of adventitious agents (all known RNA and DNA viruses, bacteria, fungi, mycoplasma etc) using the NGS (Next Generation Sequencing) platform. The virus genomic RNA was sequenced by NGS platform, and complete nucleotide sequence of MR766 strain is provided in SEQ ID NO.5 and the corresponding deduced amino acid sequence is provided in SEQ ID NO.6. Sequencing showed the intact glycosylation site in the Envelope protein, which otherwise is lost if the cells are extensively passaged in mammalian cells. Zika virus produces cytopathic effect (CPE) in Vero cells, and at the optimal Multiplicity of Infection (MoI) and harvest conditions, virus titers above $10^{8.5}$ TCID₅₀/mL or $10^{8.5}$ PFU/mL could be attained.

Example 2: Zika Virus Purification

For Zika virus culture at pilot scale, the virus culture was systematically scaled up from T-175 flasks to CS1 (cell stack 1), CS10 (cell stack 10) and CS40 (cell stack 40). Multiples of CS40 simultaneously infected with the virus at standardized MoI was used to scale up production. Use of multiples of CS40 enables quick and linear scale up to the desired volumes of production. The harvest volume from each CS40 was approximately 8-10 L. The virus was harvested at days 4-6 or whenever more than 90% CPE was achieved. Alternatively, disposable bioreactors under well standardized conditions of temperature 35°C to 37°C, pH not less than 7.0, and optimally at pH 7.4, dissolved oxygen at 45 to 75 ppm, preferably 60 rpm and an agitation of 240 to 280 rpm and optimally controlled in-flow and out-flow rate optimized according to the scale of the culture volume from 1L to 100L was used to increase the cell density and virus harvest. The viral harvest was clarified either by microfiltration or using dual filters with cut off of 1.2 µM and 0.45 µM. The clarified viral harvest was then passed through Capto Core700 column (GE healthcare Life Sciences) in phosphate buffered saline, pH 7.4. The Zika virus containing fractions in the flow through was optionally concentrated by diafiltration using either 100 kDa or 300 kDa cut off membranes. The concentrated virus fraction was used for virus inactivation. In an alternate method, the clarified viral harvest was inactivated with either BPL or formalin according to the methods described in the succeeding sections and then loaded on the column. The purity of the virus was checked on 12.5% SDS-PAGE. There was no significant difference in the yield or in purity in inactivating the virus before and after purification. The virus could also be purified using cellulose sulphate, DEAE-Sephadex CM-sephadex with salt gradient and by gel filtration on Sepharose CL-4B, ceramic hydroxyapatite column with gradient of 0.2M to 0.8M phosphate and in all cases followed by diafiltration using 100 or 300 kDa cut off membranes. The purity of the virus preparation was checked by silver staining of the virus sample in 12.5% of SDS-PAGE gel (See Figure 1). Zika virus by the aforementioned methods could be purified to high purity suitable to be used as vaccine bulk antigen. The virus could also be purified by ultracentrifugation on a 20-60% sucrose gradient using P28S rotor in Hitachi HIMAC ultracentrifuge after centrifugation at $100,000 \times g$ for 6 to 8 hours.

Example 3: Zika Virus inactivation

Zika virus sample was inactivated (killed) by various methods for use as vaccine antigens. Formalin inactivation was tested at various concentrations ranging from 1:1000 (formalin: virus, v/v) to 1: 4000 (formalin: virus, v/v) at temperature $25 \pm 5^\circ\text{C}$, more specifically at 22°C and the kinetics of virus inactivation was monitored every 24 hours for up to 10 days, and routinely the virus inactivation was carried out at $25 \pm 3^\circ\text{C}$, preferably at 22°C for 7 days. The virus inactivation was effective at all concentrations from 1:1000 v/v formalin: virus, up to 1:3500 v/v formalin: virus, at the aforementioned temperatures and time intervals. A ratio of 1:4000 v/v of formalin: virus was effective in virus inactivation at higher temperatures up to 30 to 37°C for 3 to 7 days. Formalin inactivation was effective at all the aforementioned

ratios of formalin to virus at temperatures ranging from 2-8⁰C when incubated for time intervals longer than 10 days. Hence formalin inactivation offers flexibility of virus inactivation at any temperature from 2⁰C to 37⁰C at time intervals ranging from 24 hours to more than 10 days depending upon the conditions used for inactivation. Zika virus inactivation with Beta propiolactone (BPL) was tested under various conditions. Zika virus was completely inactivated at BPL concentrations ranging from 1:1000 (BPL: virus, v/v) up to 1: 3500 (BPL: virus, v/v) at temperatures from 25±5 ⁰C for 24 to 48 hours. At higher concentration of BPL or at higher temperatures up to 37⁰C, complete inactivation was achieved in 24 hours or less, and can be used as a method for quick inactivation of the virus. Zika virus could also be inactivated at the aforementioned concentrations of BPL when incubated at 2 to 8⁰C for 3 to 7 days. A combination of BPL inactivation at 1:3500 (BPL: virus, v/v) at 22-25⁰C for 48 hours, followed by treatment with low concentrations of formalin from 1:3000 to 1: 4000 v/v of formalin: virus for 24 hours was effective in both inactivating and stabilizing the virus. Any concentration of BPL and formalin could be used for both inactivation and stabilizing the virus, as long as inactivation is complete without deleterious effect on immunogenicity. Inactivation was tested from 0.005% up to 3% final concentration of Hydrogen peroxide at 20⁰C to 25⁰C for a period 2 hours. There was no deleterious effect on the immunogenicity of the virus at lower concentrations of hydrogen peroxide with very brief exposure times within minutes but was deleterious at prolonged concentrations at higher dose ranges tested. The inactivated virus samples after exposure to different time and dose concentrations were titered for infectious virus particles if any, by TCID₅₀/mL from 5 minutes up to 6 hours at intervals of 5, 10, 20, 30 and 60 minutes and at 2, 4 and 6 hours. At higher concentrations, the virus was inactivated within seconds. At each time point, the reaction was stopped by addition of 10U/mL of catalase that rapidly hydrolyses hydrogen peroxide. The optimum concentration for inactivation was 0.01% final for duration of 60 minutes or less as determined by titration for infectious particles by TCID₅₀/mL and subsequent immunogenicity. Zika virus inactivation with hydrogen peroxide offers the flexibility of duration of exposure at different concentrations for different time points according to the concentration of virus particles in the sample.

The purified Zika virus sample was heat inactivated at temperatures 50⁰C to 65⁰C for up to 60 min. UV inactivation of the virus was carried out UV exposure at 254 nm for up to 120 minutes.

Zika virus was inactivated by gamma irradiation by exposure from 20 kGy (Kilo Gray) up to 35 kGy from a ⁶⁰Co source at the Gamma Agro Medical Processing Facility at Hyderabad. All the above inactivation methods were carried out in the presence and absence of virus stabilizing agents such as various concentrations of sugars such as sucrose, lactose, trehalose, maltose, mannose among others. The sugar alcohols used for conferring stabilizing effect were sorbitol and mannitol. The amino acids tested were selected from L-Histidine, L-Glutamic acid, L-Glycine and L-Aspartic acid and L-Glutamine and also human serum albumin and a combination of one or more of the aforementioned stabilizing agents. The most effective stabilizing agents were sorbitol at 0.5% to 2%, preferably 1.0% in combination with L-Glycine from 0.5% to 2%, preferably at 0.5%. Mannitol and L-glycine in combination was effective in stabilizing the virus sample during inactivation rather than Mannitol and L-glycine alone.

The Zika virus samples inactivated by all the aforementioned methods for use vaccine antigens were tested for completeness of inactivation by serially passaging the inactivated samples three times serially in Vero cells and testing for infectious virus at the end of

inactivation period by TCID₅₀. In addition to that, the inactivated virus sample after three serial passages *in vitro* was injected intracranially in 2-day old mice and observed for mortality or growth abnormalities for 21 days and considered completely inactivated when it showed no adverse effects *in vitro* and *in vivo* testing. No infectivity was observed with the formalin and beta-propiolactone inactivated virions at the aforementioned range of concentrations and for the various time periods tested. The inactivation kinetics of Zika virus by formalin and BPL as a representative example of one of the methods disclosed above is provided in Figure 2 (Fig.2A and Fig. 2B)

10 **Example 4: Recombinant cloning and Expression of Zika virus prME protein**

Synthetic gene of the nucleotide sequence SEQ ID NO.1 encoding the Open Reading Frame (ORF) of the prME protein of SEQ ID NO.3 of Zika virus was synthesized at GenScript, NJ, USA. The gene was PCR amplified with the primers listed below to obtain a ~ 2.1 kb fragment of SEQ ID NO.1 encoding the prME protein of SEQ ID NO.3. See Figure 3A.

15 FVFP: 5'AACTGCTCGAGGAATTCGGATCCAAC 3'

FVRP: 5' AATGGGCATGCCTGCAGGCGGCCGCTC 3'

The PCR amplified fragments was digested with EcoR1 and Not1 restriction enzymes and cloned into the EcoR1 and Not1 sites of the pFastBac plasmid vector (Life Technologies, Carlsbad, CA, USA) under the control of the polyhedron promoter by the methods described in the User's manual of Bac to Bac Baculovirus expression system ("An efficient site-specific transposition system to generate baculovirus for high-level expression of recombinant proteins, Life Technologies, USA). In brief, the method utilizes a site specific transposition of the expression cassette such as the recombinant pFastBac vector with the cloned inserts as described above into a baculovirus shuttle vector (bacmid) propagated in *E.coli*. Recombinant pFastBac vector containing one of the inserts SEQ ID NO.1 or SEQ ID NO.2 cloned under the control of the polyhedron promoter is transformed into competent cells of *E.coli* Max Efficiency DH10BacTM, that contains a baculovirus shuttle vector (bMON14272) and a helper plasmid (pMON7124) that facilitates transposition to allow efficient re-generation of the recombinant bacmid. The recombinant bacmids were selected on ampicillin, gentamicin and kanamycin containing plates by blue/white selection using blue-gal or X-gal, and IPTG. The recombinant bacmids after confirmation by PCR for the presence of the gene inserts was isolated by standard protocols described in the aforementioned User manual. About 1 µg of the bacmid DNA was used for transfection with Lipofectamine in *Spodoptera frugiperda* Sf9 insect cells (Life Technologies, Carlsbad, USA) grown in serum free insect cell medium. The methods used for transfection, isolation and titration of P1 viral stocks are exactly as described in the User's manual of Bac-to-Bac Baculovirus Expression system as given above. The P1 stocks were serially amplified twice to obtain high titer P3 stocks for expression of the recombinant prME proteins in Sf9 cells. High titer baculovirus stocks for expression of the prME protein of SEQ ID No.3 was expressed in 25 mL suspension culture of Sf9 cells and was further scaled up systematically up to 125 mL per 500 mL flask. Baculovirus infected cells from multiple flasks were harvested at 72 hours post-infection, pooled, washed once with 1 x

45 PBS, pH 7.6 and lysed in cell lysis buffer containing 10 mM phosphate, pH 7.6 with 50 mM NaCl, 1 mM PMSF and 5 mM EDTA. The cell lysate was centrifuged at 20,000 rpm for 30 minutes to remove the cell debris and the supernatant was concentrated using protein concentrators with 10 kDa cut off membrane. The concentrated sample was layered on pre-equilibrated 20% to 60% sucrose gradient and centrifuged at 100,000 x g for 6-8 hours.

Fractions containing the recombinant Membrane and Envelope protein was isolated and confirmed by Western blot (**Fig.3B**) using the rabbit MR766 polyclonal antisera. The purified recombinant protein is of the sequence of the contemporary Asian genotype of Zika virus expressed using the gene sequence SEQ ID NO.1, encoded the protein of SEQ ID No.3. The recombinant ME protein cross reacted with MR766 antibodies in Western blot and in ELISA and was formulated as vaccine antigen for testing in Balb/c mice as described in sections below.

Example 5: Vaccine Formulations

Zika virus vaccine antigen of any of the aforementioned methods in the preceding Examples was tested for immunogenicity in laboratory animals with and without adjuvants. High binding (> 95%) was observed to aluminum hydroxide (Alhydrogel® 2%, Brenntag) as the adjuvant, used at the dose range of 0.1 mg to 1.5 mg of aluminum (provided as aluminum hydroxide) per dose even when tested at the high antigen dose of 40 mcg. Binding was complete at all the concentrations of Zika virus antigens as well as vaccine combinations with CHIKV and JE antigens discussed in the succeeding sections that were used for testing in mice. Binding to aluminum hydroxide was carried out for three hours at ambient temperature. An aliquot of the formulation was centrifuged at 5000 x g for 5 min and the supernatant was tested for completeness of binding by antigen ELISA. The binding of the antigen was complete as it could not be detected in the supernatant by ELISA. The buffer for the adjuvanted formulations was 10 mM phosphate buffer, containing 154 mM NaCl, pH 7.40±0.2 and optionally containing 1% sorbitol and 0.5% L-Glycine. Other buffers used for specific formulations are mentioned below. The adjuvants listed below were tested for comparative immunogenicity and in all cases concentrations are provided per dose of the vaccine. Inactivated Zika virus antigen was tested at 10 µg per dose:

- a) Inulin (Orafti-HPX, Beneo) was tested at 0.5 mg per dose; gamma inulin was prepared by the methods described in (Cooper and Steele, 1988)
- b) A combination of aluminum hydroxide and inulin. A combination of inulin and aluminum hydroxide, algammulin was prepared at a ratio of 10:1 (10mg /mL inulin: 1 mg/mL aluminum as aluminum hydroxide) was tested at 0.5 mg per dose
- c) Muramyl di peptide (L18-MDP) (tlrl-Imdp, Invivogen) at 10 µg per dose
- d) MPL (lipid A, monophosphoryl from *Salmonella enterica*, L-6895-1 MG, Sigma Aldrich) at 25 µg per dose
- e) Combination of 0.25 mg aluminum (as aluminum hydroxide) and 25 µg of MPL per dose
- f) Oil in water emulsion (OWEM1) containing 9.75 mg of squalene (S3626-100ML, Sigma Aldrich), 11.86 mg of alpha-tocopherol (T3251-5G, Sigma Aldrich), 4.58 mg of Tween-80 (61771205001730, Merck) in 10 mM phosphate buffer, pH 7.4±0.2.
- g) Oil in water emulsion 3 (OWEM2) containing 9.75 mg squalene, 1.175 mg of tween-80, 1.175 mg Span-85 (S7135-250ML, Sigma Aldrich) in 10 mM citrate buffer, pH 7.0
- h) Poly IC (polyinosinic polycytidylic acid, potassium salt, Cat. NO. P9582-5MG, Sigma Aldrich) at 25 µg per dose
- i) Cholecalciferol (Arachitol, Abbot) at 0.75 mg per dose
- j) Resiquimod (SML0196-10MG, Sigma Aldrich) + Poly IC, 25 µg each
- k) Resiquimod (25 µg) + Oil in water emulsion 2 containing 9.75 mg squalene, 1.175 mg of tween-80, 1.175 mg Span-85 (S7135-250ML, Sigma Aldrich) in 10 mM citrate buffer, pH 7.0

1) Aluminum 0.25 mg and 0.5 mg per dose provided as aluminum hydroxide

5 All the above formulations elicited high level of neutralizing antibodies and the results are depicted in **Figure 4**. The individual components of the aforementioned adjuvants and any of their analogues, derivatives, side chain substitutions and any modifications of any of the above components at varying concentrations can be used as non-toxic vaccine adjuvant components as long as they have immunopotentiating effect. Formalin inactivated and recombinant Zika vaccine antigens as described in the aforementioned sections each at a concentration of 10 µg per dose was lyophilized in combination with either of the following
10 excipients: 1% mannitol and 0.5% Glycine, 5% sucrose and 1% trehalose, 5% sucrose and 1% maltose and 2% mannitol and 0.5% Glycine. The dry lyophilized formulation could be easily reconstituted in aqueous solution with water, normal saline and 10 mM phosphate buffered saline, pH 7.4±0.2. The stability of the formulation was tested at 37°C for two weeks. No change in the cake characteristics was observed indicating the stability of the
15 formulations. The moisture content was below 1%.

Example 6: Effect of stabilizing agents

The stability of the formalin inactivated vaccine bulk for use as non-adjuvanted vaccine antigen was tested for stability with the following concentration of stabilizing agents: a) 2% sorbitol and 1% L-glycine; b) 1% sorbitol and 0.5 % L-glycine c) 1% mannitol and 0.5% L-glycine; d) 1% mannitol and 0.5% L-glutamic acid e) 1% sorbitol and 0.5% L-glycine, 1% human serum albumin. Stability testing was done at 37°C for 2 weeks and the antigen concentration was tested by ELISA before and after exposure at 37°C. 1 µg and 10 µg of the non-adjuvanted formulation with 1% sorbitol and 0.5% L-Glycine was tested for
20 immunogenicity in Balb/c mice as discussed in the succeeding sections.
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Example 7: Potency testing of Vaccine formulations in animal models

Zika vaccine antigen inactivated by the aforementioned methods was tested in Balb/c mice in dose ranges from 0.125 µg up to 40 µg of antigen per dose with 0.25 mg aluminum per dose (as aluminum hydroxide) in a volume of 100 µL (injected in two sites at 50 µL per site) by intramuscular route on days 0, 14, 28. Initial testing on the effect of aluminum (provided as aluminum hydroxide) showed that alum adsorbed vaccine gave higher titer of neutralizing antibodies than non-adjuvanted vaccine. About 1 and 10 µg of inactivated vaccine antigen without alum contained 1% sorbitol and 0.5% L-glycine as the excipients to confer stability
30 to the vaccine antigens. Blood was drawn from retro-orbital sinus on days 13, 21 and 35 for estimation of neutralizing antibody titers by PRNT₅₀, total Ab titer by ELISA, Ab avidity and cytokine profiles. Blood withdrawal and testing after each dose gave data on the potency and safety of single, two doses and three doses of the vaccine preparations. The animals were each challenged on day 36 with 10e5 PFU of Zika virus by intravenous route. The blood samples were monitored for up to 7 days at 24 hour intervals for formalin groups and at two
40 points at 48 hours and 96 hours for BPL inactivation groups for protection against viremia by TCID₅₀ (50% Tissue Culture Infectious Dose) and the virus titers if any, were expressed as TCID₅₀/mL. Animal challenge studies showed complete protection from viremia in 1 µg to 40 µg of the dose groups tested. Hence the BPL and formalin inactivated vaccine
45 formulations were further tested at 0.5 µg, and at 0.25 µg 0.125 µg per dose by the IM route in Balb/c mice and were found to be immunogenic even at low dilutions. For the alum adjuvanted formulations, 0.25 mg of aluminum (as aluminum hydroxide) per dose was used as the placebo control and for non-adjuvanted formulations, 10 mM phosphate buffer containing 154 mM NaCl, 1% sorbitol and 0.5% L-Glycine, pH 7.40 was used as the vehicle

control. All the formalin and BPL inactivated formulations elicited high level of neutralizing antibodies and protected against viremia as depicted in **Fig.5A, Fig. 5B and Figure 6**. Antigen only formulations also elicited high level of neutralizing antibodies and was protected from virus challenge. Recombinant prME protein expressed in insect cells was formulated at two doses of 10 and 20 µg per dose with 0.25 mg aluminum (as aluminum hydroxide) per dose in Balb/c (8 nos) and injected intramuscularly at day 0 and day 21 elicited neutralizing antibodies and the data is provided in Table 1. Gamma irradiated and Hydrogen peroxide inactivated Zika virus antigen at dose concentration of 10 µg and formulated with 0.25 mg aluminium (as aluminium hydroxide) per dose was injected by IM route in Balb/c mice at day 0 and day 21 and blood was withdrawn on day 28 for estimation of neutralizing antibodies by PRNT₅₀. Formalininactivated virus antigen at 10 µg was formulated with each of the adjuvants disclosed in Example 5 and was injected intramuscularly in 4-6 week old Balb/c mice (5 nos per dose group) and the blood was drawn at 21 days after vaccine administration for estimation of neutralizing antibodies and cytokines. Control groups was included for each of the adjuvants and no neutralizing antibodies (≤ 10 by PRNT₅₀) could be detected and the data is not shown. Neutralizing antibody titers by PRNT₅₀ of the different adjuvanted formulations, used pooled sera from each group is presented in **Figure 4**. High level of neutralizing antibodies were elicited by the aforementioned adjuvanted formulations.

A combination vaccine of arbovirus antigens were prepared at the following concentrations and tested in Balb/c mice: a) 10 µg formalin inactivated Zika virus antigen, 20 µg of BPL inactivated Chikungunya virus antigen and 6 µg of formalin inactivated JE antigen in a trivalent vaccine combination b) 10 µg formalin inactivated Zika virus antigen and 20 µg of BPL inactivated CHIKV virus antigen c) 10 µg of formalin inactivated Zika virus antigen and 6 µg of JE virus antigen. All the above vaccine combinations were tested with 0.25 mg aluminum (as aluminum hydroxide) per dose in Balb/c mice (8 nos each) with appropriate controls that included either of the aforementioned antigens alone, and also control animals that received equivalent amount of alum. The animals were boosted at 14 and at 21 days after the first immunization. Blood was collected at 7 days after the last booster injection. The sera samples were used for estimation of neutralizing antibody by PRNT₅₀ for Zika, CHIKV and JEV. The buffer used in all the formulations was 10 mM phosphate buffer, pH 7.2 to 7.6 containing 154 mM NaCl. All the methods disclosed above are applicable to any genotype/genotypic variants/serotypes and strains of Chikungunya virus, Zika virus and Japanese encephalitis viruses. See Table 1 for the results.

Table 1: Neutralizing antibodies elicited by various antigenic formulations as disclosed in the Examples.

Test Groups	Neutralizing antibody titers as Log ₁₀ PRNT ₅₀		
	Zika a	CHIKV	JE
Recombinant Zika prME – 10 µg x 2 doses	2.8	-	-
Recombinant Zika prME – 20 µg x 2 doses	3.22	-	-
Hydrogen peroxide inactivated Zika antigen – 10 µg x 2 doses	2.6	-	-
Gamma irradiated Zika antigen 10 µg x 2 doses	2.71	-	-
Zika alum adsorbed – 10 µg x3 doses	3.06	-	-
Chikungunya alum adsorbed –	-	2.808	-

20 µg x 3 doses			
JE alum adsorbed – 6 µg x 3 doses	-	-	3.06
Zika (10 µg) + CHIKV (20 µg) x 3 doses	2.95	2.68	-
Zika (10 µg) + JE (6 µg) x 3 doses	2.80	-	3.28
Zika (10 µg) + CHIKV(20 µg) + JE (6 µg) x 3 doses	2.79	2.63	3.21

Table 1 Legend: Purified recombinant prME antigen of Zika virus and the hydrogen peroxide inactivated and gamma irradiated Zika virus antigens formulated with 0.25 mg of aluminum per dose elicited neutralizing antibodies in Balb/c mice. The titers are expressed as Log₁₀PRNT₅₀ values. The vaccine combinations elicited neutralizing antibodies when two or more antigens were administered in a single formulation, and no significant antigenic interference was observed between JE, Zika and CHIKV viruses

Example 8: Passive immunization studies

The proof of concept that neutralizing antibodies are important immune correlates of protection against Zika virus infection was demonstrated by single injection of rabbit polyclonal Zika antisera with known titer, About 200 µL of antisera diluted 1:1 with PBS was injected intraperitoneally in Balb/c mice and challenged 8-24 hours later with 10⁵ PFU of Zika virus by intravenous route in a volume of 100 µL. Equal no. of control animals received PBS, pH 7.4 and received the virus injection as the test animals. Blood was collected at 24, 48, 72, 96 and 144 hours post virus challenge for detection of viremia in both the group of animals. Passive immunization offered complete protection against viremia and infectious virus could not be detected by TCID₅₀. See Figure 7. Viremia was detected in the control animals that persisted up to 6 days after virus challenge. Zika antibodies could be used as a therapeutic to ameliorate, eradicate or prevent Zika virus infections.

Example 9: Assays for Neutralizing Antibody Titers

Animal sera from all the aforementioned vaccine testing in mice described in Example 7 which include all the monovalent Zika vaccines inactivated with different inactivating agents and formulated with different adjuvants, vaccine antisera from dose ranging studies as well combination vaccines with CHIKV and JEV described in the preceding sections were assayed for neutralizing antibodies by 50% Plaque Reduction Neutralization Test (PRNT₅₀) by standardized procedures. Briefly, one day prior to the assay, 6-well plates were seeded with 2.5 x 10³ Vero cells (ATCC CCL-81) per well and the plates were incubated at 37°C in a 5% CO₂ incubator. To 4-fold dilutions of the sera samples in MEM containing equal volume of the standardized Zika virus strain (10⁵ pfu/mL) was added and incubated at 37°C with 5% CO₂ for 90 min. The cells were washed twice with 1 x PBS pH 7.4 (10 mM phosphate with 150 mM NaCl) and 0.30 ml of each dilution of the serum-virus mixture was added to the corresponding well and incubated for 90 min at 37°C in a 5% CO₂ incubator. Each assay was carried out in triplicates. The cells were overlaid with 2 ml of 0.85% methyl cellulose in MEM with 1% penicillin-streptomycin and 1% L-glutamine. The plates were incubated at 37°C in a 5% CO₂ incubator for 4 days. At the end of incubation, the plaques were fixed with 10% formalin, washed with 1 x PBS, pH 7.4 and were visualized with 0.1% crystal violet. The highest dilution of serum causing 50% reduction in the number of plaques formed by the control virus sample was estimated as the PRNT₅₀ titer. Anti-CHIKV and anti-JE antibodies from the vaccine combinations were also estimated PRNT₅₀. All the aforementioned vaccine antigens elicited high level of neutralizing antibodies as depicted in **Figures 5 and 6**

Example 10: Zika virus cross neutralization studies

Formalin inactivated vaccine antisera cross neutralized the homologous MR766 virus strain of the African genotype and FSS13025 Zika virus strain (GenBank Acc No. JN860885) of the Asian genotype with EQUAL efficiency with PRNT₅₀ titers of 18105 and 18325 against MR766 and FS13025 strains respectively. (The study BS-3018 was contracted to IBT Bioservices, Gaithersburg, MD, USA). Briefly, both the MR766 and the FS13025 Zika virus strains were diluted to ~ 250 PFU in serum-free medium. Both the vaccine antisera and control sera (placebo) were serially diluted in two-fold dilutions. The virus samples were mixed 1:1 with serially diluted sera samples and incubated at 37°C for 2 hours. Vero cells seeded in 24-well plates were infected with the dilutions for 1 hour and 0.85% methyl cellulose was added to each well and incubated for 3 days. Cells were fixed and analyzed by plaque assay. The plates were scanned and the plaque counts were used to calculate the PRNT₅₀ titers using a 4PL curve fit. Hence the method of vaccine antigen preparation, formulation and testing are entirely applicable across any genotype of Zika virus as the vaccine with one genotype 100% cross neutralizes the heterologous strain and this also proves that no serotypes of Zika virus exists and that inactivated vaccine of Zika using any strain will be equally protective and potent as vaccine prepared using any genotype, and genotypic variant or indeed any Zika virus strain. This fact was further corroborated when the antibodies raised against the recombinant protein expressed as prME (protein of SEQ ID No.3) in insect cells cross neutralized the MR766 virus with high efficiency. The protein of SEQ ID No.3 is derived from the prME of the African genotype of Zika virus strain H/PF/013, which is the more contemporary strain of the Asian genotype. Cross neutralization of the vaccine antisera of the homologous MR766 strain of nucleotide SEQ ID NO.5 encoding the complete ORF of SEQ ID NO.6 and the heterologous FSS13025 of the SEQ ID No.7 encoding the complete ORF of SEQ ID NO. 8 is depicted in **Figure 8A and Fig. 8B**.

Example 11: Antibody ELISA

Briefly, Zika virus antigen was coated at the standardized concentration in coating buffer in 96-well plates overnight at 2 to 8°C. The plate contents were discarded and the wells were blocked with blocking buffer and washed extensively before adding the vaccine antisera at serial dilutions. Each vaccine antisera was assayed in triplicates. The plates were incubated for 90 min at 37°C, before adding secondary antibody (anti mouse-IgG HRPO conjugate) diluted 1:2500 in antibody diluent buffer. Each of the wells were washed five times with washing buffer (PBST, pH 7.4) and three times with PBS (pH 7.4), 30 seconds each. About 100 µl /well of freshly prepared substrate solution was added and incubated at ambient temperature for 10 minutes for color development. The color development was stopped by addition of 50 µL /well stop solution. Absorbance was read at 492 nm and the results recorded. For each assay, antigen blank, primary and secondary antibody blanks were included as controls. Seroconversion cut off value = pre-exposure average titer + (3 x standard deviation). The end point dilution of positively seroconverted sample which shows a titer equivalent to the pre-exposure level titer was identified. Reciprocal of the penultimate dilution of end point of a positively seroconverted sample was interpreted as the antibody endpoint titer. Antibody titers to both BPL inactivated and formalin inactivated Zika vaccine formulations were higher with aluminum hydroxide than with antigens alone. Vaccine formulations of the formalin inactivated vaccine at all doses (**Fig. 9A-9C**) and all doses of BPL inactivated vaccine (data not shown) elicited high level of antibodies, after each dose of vaccine administration confirming that vaccine can be administered as a single dose or two or more doses for eliciting a robust immune response against Zika virus.

Example 12: Antibody avidity

The quality of antibody responses to the vaccine was estimated by antibody avidity assays. The antigen-antibody binding avidities are the degree of affinity maturation in the B-cells. Higher antibody avidities correlate with neutralizing antibodies in several vaccine studies. Prior to determination of avidity index, titrations with sodium isothiocyanate (NaSCN) from 0 M to 6 M concentration in 0.25 M steps from 0 to 2.0 M were performed. After addition and incubation of primary antisera to the antigen coated plates, the plates were incubated with graded concentrations NaSCN for 15 min with intermittent shaking, washed and developed as in regular ELISA. The optical densities obtained at each of the concentrations were plotted. The highest OD (A) was plotted and halved (A/2), and the distance between the OD curves at A/2 was measured as the NaSCN shift value. The NaSCN shift was higher after the first booster dose compared to the prime dose and remained static or marginally increased further after second booster dose administration indicating that high affinity antibodies developed over time and with booster injections. A reference point in the ELISA titration was taken calculation of avidity index, (AI) which is the ratio of antibody concentration (measured by absorbance) in ELISA of serum samples treated with and without the chaotropic agent NaSCN. Even at the lowest single dose concentration of 1 µg of formalin inactivated Zika vaccine, antibodies with high affinity binding to the antigen was detected, indicating the vaccine is potent even at low concentrations of the vaccine antigen (See **Figure 10**).

Example 13: Cytokine profiling

Both Th1 and Th2 cytokines were estimated in mice sera after administration of two doses of the formalin inactivated Zika antigen formulated with different adjuvants including aluminium hydroxide and in antigen only controls for comparison. The Mouse ELISA kit - Th1 / Th2 (Catalog No. 88-7711-44, eBioscience) was used for the estimation of IL-2, IFN gamma, IL-4 and IL-10 by methods exactly as per the kit protocols using the standards provided in the kit. The concentration of the cytokines are expressed in pg/mL. The results for Th1 cytokine levels are depicted in **Figure 11A and Fig.11B** and Th2 cytokines in **Fig12A and Fig.12B**.

Example 14: Estimation of virus titers

The amount of infectious virus particles in the upstream and downstream bioprocess samples, Zika virus titers for animal challenge studies were measured by TCID50 (50% Tissue Culture Infectious Dose) assay. This assay measures the dilution of the virus sample that generates cytopathic effect (CPE) in 50% of the cells. Vero cells were seeded in 96-well microplates and incubated in 5% CO₂ at 37°C overnight. The cells were infected with 10-fold serial dilutions of virus sample, followed by incubation for 5 d in 5% CO₂ at 33°C. The cells were visually inspected for CPE and the TCID50 titer was calculated according to the method of Reed and Muench (Reference). The results are presented as a log₁₀ titer (10xTCID50 units/mL). Alternatively plaque assays were used and the titers were expressed as plaque forming units, PFU /mL.

REFERENCES

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We claim:

1. A stable vaccine composition comprising one or more arbovirus antigens selected from Zika virus, Chikungunya virus and Japanese encephalitis virus, said antigens being formulated with or without an adjuvant in pharmaceutically acceptable buffer and optionally a preservative, wherein the vaccine composition elicits protective immune response to each of the viruses in mammals.
2. The vaccine composition as claimed in claim 1, wherein the said antigens are inactivated whole virion (virus) antigens.
3. The vaccine composition as claimed in claim 1, wherein the said antigens are purified recombinant antigens.
4. The vaccine composition as claimed in claim 1, wherein the said antigens are prepared using Vero cells as cell substrate by adapting the viruses to Vero cells.
5. The vaccine composition as claimed in claim 1, wherein said antigens are purified and concentrated antigens obtained from one or more methods selected from:
 - a. ultracentrifugation;
 - b. density gradient centrifugation;
 - c. clarification of the viral harvest using membrane filtration, followed by purification by column chromatography; and
 - d. tangential flow filtration using membranes with cut off from 100 kDa to 300 kDa, wherein tangential filtration is carried out either before or after virus inactivation.
6. The vaccine composition as claimed in claim 5, wherein said purification by column chromatography comprises gel filtration, mixed mode resin column chromatography, ion exchange column chromatography, affinity matrix chromatography and hydrophobic interaction chromatography.
7. The vaccine composition as claimed in claim 6, wherein the column chromatography elutes majority of the virus antigen in the flow through using mixed mode size exclusion matrix.
8. The vaccine composition as claimed in claim 2, wherein the said whole viruses are inactivated by at least one or more of a chemical inactivating agent, a physical inactivating agent, an irradiating agent.
9. The vaccine composition as claimed in claim 8, wherein the inactivation is carried out before or after purification of the viruses.
10. The vaccine composition as claimed in claim 9, wherein the viruses are inactivated by chemical inactivating agent selected from formalin (formaldehyde), beta propiolactone (BPL) and hydrogen peroxide.
11. The vaccine composition as claimed in claim 10, wherein the viruses are inactivated by any one of the following methods selected from:
 - a. Formalin treatment at any concentration ranging from 1: 500 up to 1: 4000 v/v of formalin: virus, at 8⁰C to 37⁰C, preferably 25±3⁰C, for at least 1 to 7 days;

- b. Formalin treatment at any concentration ranging from 1:500 up to 1: 4000 v/v of formalin: virus, at 2⁰C to 8⁰C for at least 10 to 30 days;
 - c. Beta propiolactone (henceforth BPL) at any concentration ranging from 1:500 up to 1: 4000 v/v of BPL: virus, for at least 24 to 48 hrs at temperatures ranging from 8⁰C to 30⁰C, preferably 25±3⁰C, for 48 hours;
 - d. Beta propiolactone at any concentration ranging from 1: 500 up to 1:4000 (BPL: virus, v/v), at 2⁰C to 8⁰C for at least 3-7 days;
 - e. A combination of BPL and formalin at any of the aforementioned conditions, preferably BPL inactivation at 1:3000 (BPL: virus, v/v) for 24 hours followed by formalin inactivation at 1: 3000 (formalin: virus, v/v) for 24to 48 hours at 15⁰C to 30⁰C, preferably 25±3⁰C;
 - f. Hydrogen peroxide at any concentration from 0.1 to 3%, preferably 0.1 to 1% at any temperature from 20 - 30⁰C for 5 minutes to 120 minutes.
12. The vaccine composition as claimed in Claim 8, wherein the inactivation of the viruses by irradiating agent comprises inactivation by gamma irradiation by exposure from 10 kGy (Kilo Gray) up to 35 kGy, preferably 25 kGy to 30 kGy from a ⁶⁰Co source.
13. The vaccine composition as claimed in claim 8, wherein inactivation of the viruses by irradiating agent comprises inactivation by UV irradiation by exposure to 254 nm for 30 – 60 minutes.
14. The vaccine composition as claimed in claim 8, wherein the virus is inactivated by heat treatment at a temperature between 50⁰C to 65⁰C for 30 min up to 2 hrs.
15. The vaccine composition as claimed in claim 1, wherein the buffer is selected from the list comprising of phosphate buffer, citrate buffer, phosphate citrate buffer, borate buffer, tris(hydroxymethyl)aminomethane (Tris) containing buffer, succinate buffer, buffers containing glycine or histidine as one of the buffering agents.
16. The vaccine composition as claimed in claim 15, wherein phosphate buffer is sodium phosphate buffer at concentration of 5 mM up to 200 mM of phosphate ions of any pH between 6.50 to pH 9, and optionally containing sodium chloride at a concentration of 50 to 200 mM.
17. The vaccine composition as claimed in claim 1, wherein the buffer maintains the pH in a liquid composition above pH 6.5, preferably above pH 7.0 throughout the bioprocess from viral culture up to preparation of purified inactivated virus bulk.
18. The vaccine composition as claimed in claim 8, wherein the inactivation is carried out in the presence of a stabilizing agent selected from lactose, sucrose, trehalose, maltose, mannose, iso-maltose, raffinose, stachyose, lactobiose, sorbitol, mannitol, lactobionic acid, dextran, L-glycine, L-histidine, L-glutamic acid, L-aspartic acid and human serum albumin or combinations thereof.
19. The vaccine composition as claimed in claim 18, wherein the stabilizing agent is selected from:
- a. 2% sorbitol and 1% L-glycine;
 - b. 1% sorbitol and 0.5 % L-glycine;
 - c. 1% mannitol and 0.5% L-glycine;

- d. 1% mannitol and 0.5% L-glutamic acid; and
 - e. 1% sorbitol and 0.5% L-glycine, 1% human serum albumin.
20. The vaccine composition as claimed in 8, wherein the inactivation of Zika virus comprises inactivation of any genotype/strain, live attenuated Zika virus, deactivated virus, virus like particles, chimeric virus particles that carry any Zika virus antigens particularly the E protein in any heterologous virus backbone, in vectored vaccines and infectious synthetic virus particles derived *in vitro* or *in vivo* using the sequence of any Zika virus genome.
21. The vaccine composition as claimed in claim 3, wherein the purified recombinant Zika virus comprises antigens of Zika virus comprising the envelope (E) protein, membrane (M) protein and optionally the non-structural 1 (NS1) protein as vaccine antigens for eliciting immune response for prophylaxis of Zika virus infections.
22. The vaccine composition as claimed in claim 21, wherein the Zika virus has the structural protein sequences as disclosed in SEQ. ID No. 3 and SEQ. ID No.4 corresponding to nucleotide sequences of SEQ ID. No. 1 and SEQ ID No.2 respectively, for use as vaccine antigens against Zika virus infections caused by genotypes or variants thereof.
23. The vaccine composition as claimed in claim 3, wherein the Recombinant DNA constructs comprises a (i) vector (ii) at least one nucleic acid fragment corresponding to SEQ ID NO.1 or SEQ ID NO. 2 encoding the amino acid sequence of the proteins of SEQ ID NO.3, SEQ ID NO.4 respectively which is applicable to any Zika virus protein sequences that share at least 70% amino acid identity to the aforementioned SEQ ID NO. 3 and SEQ ID NO.4.
24. The vaccine composition as claimed in claim 23 comprising a recombinant DNA construct, wherein the vector is an eukaryotic plasmid vector being cloned in a eukaryotic host such as baculovirus for expression in insect cells as virus like particles (VLPs).
25. The vaccine composition as claimed in claim 22, wherein the recombinant proteins of the viruses are obtained by the process comprising the steps of:
- a. transfecting the recombinant plasmid DNA in insect cells;
 - b. harvesting the cells and isolating the recombinant protein therefrom;
 - c. purifying the protein by a method selected from ion exchange chromatography, gel filtration, affinity chromatography, hydrophobic column chromatography, mixed mode resin chromatography, diafiltration, ultracentrifugation, density gradient centrifugation and fractionation with salt.
26. The vaccine composition as claimed in claim 1, wherein the structural antigens of the viruses are expressed in prokaryotic and eukaryotic expression system including baculovirus mediated expression in insect cells.
27. The vaccine composition as claimed in claim 1, wherein the composition is obtained by a process wherein neutralizing antibodies are largely elicited against the Envelope protein such as in optimally inactivated virus, live attenuated virus, deactivated virus, DNA vaccine, virus like particles, chimeric virus particles that display the Zika virus

E protein in any heterologous virus backbone such as in vectored vaccines and synthetic virus particles derived from any Zika virus genomic RNA sequence.

28. The vaccine composition as claimed in claim 1, further comprising an adjuvant.
29. The vaccine composition as claimed in claim 28, wherein the adjuvant is selected from the group consisting of a) aluminum salts comprising aluminum hydroxide, aluminum phosphate, aluminum sulphate phosphate; b) inulin; c) algammulin which is a combination of inulin and aluminium hydroxide; d) monophosphoryl lipid A (MPL); e) resiquimod; f) muramyl dipeptide (MDP); g) N-glycolyl dipeptide (GMDP); h) polyIC; i) CpG oligonucleotide; j) aluminum hydroxide with MPL; k) water in oil emulsion; l) oil in water emulsion that contains one or more of the following constituents: squalene or its analogues or any pharmaceutically acceptable oil, tween-80, sorbitan trioleate, alpha-tocopherol, cholecalciferol and aqueous buffer, or analogues and derivatives of the molecules thereof, used individually or in combinations.
30. The vaccine composition as claimed in claim 29, wherein the composition comprises aluminum hydroxide in a concentration range of 0.1 mg to 1.5 mg of aluminum per vaccine dose, preferably 0.25 mg to 0.5 mg aluminum per vaccine dose.
31. The vaccine composition as claimed in claim 29, wherein the adjuvant confers mucosal immunity and systemic immunity when administered in mammals.
32. The vaccine composition as claimed in claim 1, wherein the composition with the virus antigens is administered at any dose ranging from 0.125 µg to 100 µg per dose with or without an adjuvant, either as a single dose or in two or more doses to elicit an immune response in a mammal.
33. The vaccine composition as claimed in claim 1 comprising Zika virus and Japanese encephalitis virus antigens in a combination vaccine that elicits protective immune response in mammals against each of the viruses.
34. The vaccine composition as claimed in claim 33, wherein the virus inactivated antigens are present in the combination vaccine at concentrations ranging 5 µg to 50 µg of each antigen in a pharmaceutically acceptable formulation without an adjuvant, or with an adjuvant.
35. The vaccine composition as claimed in claim 34, wherein the adjuvant is selected from the group consisting of a) aluminum salts comprising aluminum hydroxide, aluminum phosphate, aluminum sulphate phosphate; b) inulin; c) algammulin which is a combination of inulin and aluminium hydroxide; d) monophosphoryl lipid A (MPL); e) resiquimod; f) muramyl dipeptide (MDP); g) N-glycolyl dipeptide (GMDP); h) polyIC; i) CpG oligonucleotide; j) aluminum hydroxide with MPL; k) water in oil emulsion; l) oil in water emulsion that contains one or more of the following constituents: squalene or its analogues or any pharmaceutically acceptable oil, tween-80, sorbitantriolate, alpha-tocopherol, cholecalciferol and aqueous buffer, or any of the analogues and derivatives of the molecules thereof, used individually or in combinations.

36. The vaccine composition as claimed in claim 35, wherein the adjuvant is aluminium hydroxide with 0.25 mg to 1.0 mg of aluminium content per vaccine dose.
37. The vaccine composition as claimed in claim 36, wherein the virus antigens are present in a combination vaccine at concentrations ranging from 5 µg to 50 µg of each antigen in a pharmaceutically acceptable formulation without an adjuvant, or with an adjuvant.
38. The vaccine composition as claimed in claim 37, wherein the adjuvant is selected from the group consisting of a) aluminum salts comprising aluminum hydroxide, aluminum phosphate, aluminum sulphate phosphate; b) inulin; c) algammulin which is a combination of inulin and aluminium hydroxide; d) monophosphoryl lipid A (MPL); e) resiquimod; f) muramyl dipeptide (MDP); g) N-glycolyl dipeptide (GMDP); h) polyIC; i) CpG oligonucleotide; j) aluminum hydroxide with MPL; k) any water in oil emulsion; l) any oil in water emulsion that contains one or more of the following constituents: squalene or its analogues or any pharmaceutically acceptable oil, tween-80, sorbitantriolate, alpha-tocopherol, cholecalciferol and aqueous buffer, or any of the analogues and derivatives of the molecules thereof, used individually or in combinations.
39. The vaccine composition as claimed in claim 38, wherein the adjuvant is aluminium hydroxide at 0.25 mg to 1.5 mg of aluminium content per vaccine dose.
40. The vaccine composition as claimed in claim 1 comprising Zika virus, Chikungunya virus and Japanese encephalitis virus antigens in a combination vaccine that elicits protective immune response in mammals against each of the viruses.
41. The vaccine composition as claimed in claim 40, wherein the virus antigens are present in a combination vaccine at concentrations ranging from 5 µg to 50 µg of each antigen in a pharmaceutically acceptable formulation without an adjuvant, or with an adjuvant.
42. The vaccine composition as claimed in claim 41, wherein the adjuvant is selected from the group consisting of a) aluminum salts comprising aluminum hydroxide, aluminum phosphate, aluminum sulphate phosphate; b) inulin; c) algammulin which is a combination of inulin and aluminium hydroxide; d) monophosphoryl lipid A (MPL); e) resiquimod; f) muramyl dipeptide (MDP); g) N-glycolyl dipeptide (GMDP); h) polyIC; i) CpG oligonucleotide; j) aluminum hydroxide with MPL; k) any water in oil emulsion; l) any oil in water emulsion that contains one or more of the following constituents: squalene or its analogues or any pharmaceutically acceptable oil, tween-80, sorbitantriolate, alpha-tocopherol, cholecalciferol and aqueous buffer, or any of the analogues and derivatives of the molecules thereof, used individually or in combinations.
43. The vaccine composition as claimed in claim 42, wherein the adjuvant is aluminium hydroxide at 0.25 mg to 1.0 mg of aluminium content per vaccine dose.
44. The vaccine composition as claimed in claim 1, wherein the preservative is 2-phenoxyethanol in a concentration ranging from 2.5 to 5 mg/mL.
45. The vaccine composition as claimed in claim 1, when administered in a single dose or in two or more doses in mammals elicits both Th1 and Th2 immune response

against any of the arbovirus antigens comprising Zika Virus, Chikungunya virus and Japanese Encephalitis virus and is suitable for administration to humans.

46. A method for preparation of a vaccine composition comprising one or more arbovirus antigens selected from Zika virus, Chikungunya virus and Japanese encephalitis virus, the method comprising one or more steps of inactivation, producing recombinant protein, expressing structural antigens, purification and concentration of the virus antigen wherein said purification and concentration of Zika, Chikungunya and Japanese Encephalitis virus comprises one or more steps selected from:
 - a. ultracentrifugation;
 - b. density gradient centrifugation;
 - c. clarification of the viral harvest using membrane filtration;
 - d. purification by column chromatography;
 - e. tangential flow filtration using membranes with cut off from 100 kDa to 300 kDa, wherein tangential filtration is carried out either before or after virus inactivation.
47. The method as claimed in claim 46, wherein the column chromatography method comprises gel filtration, mixed mode resin column chromatography, any ion exchange column chromatography, affinity matrix chromatography and hydrophobic interaction chromatography.
48. The method as claimed in claim 46, wherein the column chromatography elutes majority of the virus antigen in the flow through using mixed mode size exclusion matrix.
49. The method as claimed in claim 46, wherein the viruses are inactivated by one or more inactivating agents selected from a chemical inactivating agent, a physical inactivating agent and an irradiating agent.
50. The method as claimed in claim 46, wherein inactivation of the viruses is carried out before or after purification of the viruses.
51. The method as claimed in claim 50, wherein the viruses are inactivated by chemical inactivating agent selected from formalin (formaldehyde), beta propiolactone (BPL) and hydrogen peroxide.
52. The method as claimed in claim 49, wherein the virus bulk are inactivated by any one of the following methods selected from:
 - a. Formalin treatment at any concentration ranging from 1: 500 up to 1: 4000 v/v of formalin: virus, at 8⁰C to 37⁰C, preferably 25±3⁰C, for at least 1 to 7 days;
 - b. Formalin treatment at any concentration ranging from 1:500 up to 1: 4000 v/v of formalin: virus, at 2⁰C to 8⁰C for at least 10 to 30 days;
 - c. Beta propiolactone (henceforth BPL) at any concentration ranging from 1:500 up to 1: 4000 v/v of BPL: virus, for at least 24 to 48 hrs, if not more, at temperatures ranging from 8⁰C to 30⁰C, preferably 25±3⁰C, for 48 hours;
 - d. Beta propiolactone at any concentration ranging from 1: 500 up to 1:4000 (BPL: virus, v/v), at 2⁰C to 8⁰C for at least 3-7 days;
 - e. a combination of BPL and formalin at any of the aforementioned conditions, preferably BPL inactivation at 1:3000 (BPL:virus, v/v) for 24 hours followed by

formalin inactivation at 1: 3000 (formalin: virus, v/v) for 24 to 48 hours at 15°C to 30°C, preferably 25±3°C ;

f. Hydrogen peroxide at any concentration from 0.1 to 3%, preferably 0.1 to 1% at any temperature from 20 - 30°C for 5 minutes to 120 minutes.

53. The method as claimed in claim 49, wherein the viruses are inactivated by gamma irradiation by exposure from 20 kGy (Kilo Gray) up to 35 kGy, preferably 25 kGy to 30 kGy from a ⁶⁰Co source.

54. The method as claimed in claim 49, wherein the viruses are inactivated by UV irradiation by exposure to 254 nm for 30 – 60 minutes.

55. The method as claimed in claim 49, wherein the viruses are inactivated by heat treatment from 50°C to 65°C for 30 min up to 2 hrs, preferably, 65°C for 1 hr.

56. The method as claimed in claim 49, wherein the inactivation is carried out in the presence of stabilizing agent selected from lactose, sucrose, trehalose, maltose, mannose, iso-maltose, raffinose, stachyose, lactobiose, sorbitol, mannitol, lactobionic acid, dextran, L-glycine, L-histidine, L-glutamic acid, L-aspartic acid and human serum albumin or combinations thereof.

57. The method as claimed in claim 56, wherein the stabilizing agent is selected from:

- a. 2% sorbitol and 1% L-glycine;
- b. 1% sorbitol and 0.5 % L-glycine;
- c. 1% mannitol and 0.5% L-glycine;
- d. 1% mannitol and 0.5% L-glutamic acid; and
- e. 1% sorbitol and 0.5% L-glycine, 1% human serum albumin.

58. The method as claimed in claim 46, wherein the method of producing the recombinant protein comprises the steps of:

- a. transfecting recombinant plasmid DNA in insect cells;
- b. harvesting the cells and isolating the recombinant protein therefrom;
- c. purifying the protein by at least one of the methods comprising of ion exchange chromatography, gel filtration, affinity chromatography, hydrophobic column chromatography, mixed mode resin chromatography, diafiltration, ultracentrifugation, density gradient centrifugation, fractionation with salt.

59. The method as claimed in claim 46, wherein the method of expressing the structural antigens of the virus comprising expression system is prokaryotic or eukaryotic expression system including baculovirus mediated expression in insect cells.

Dated this 30th day of January 2019.



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ABSTRACT

VACCINE COMPOSITIONS

The present disclosure provides vaccine compositions for prophylaxis and treatment of Zika virus infections comprising Zika virus antigens in immunogenic compositions, and in combination of Zika antigens with one or more arbovirus antigens such as Chikungunya virus and Japanese encephalitis virus antigens, methods of preparation and production of such compositions for use as vaccines for eliciting immune response in mammals against the above mentioned pathogens.

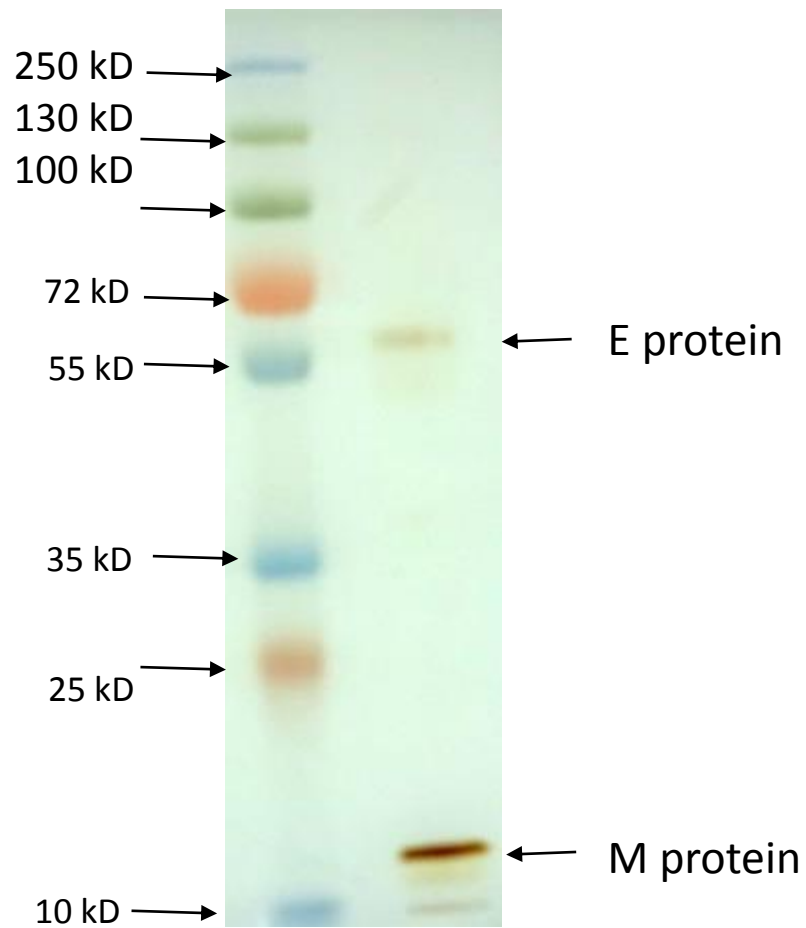


Figure 1