routes that remain undetermined (Faria *et al.* (2016) Science. 352(6283):345-349). The virus may be transmitted zoonotically by *Aedes aegypti*, *A. albopictus*, and possibly by *A. hensilli* and *A. polynieseinsis* (Weaver *et al*. (2016) Antiviral Res. 130:69-80). Additionally, it is thought that other vectors for transmitting the virus may exist, and the virus may be transmitted by blood transfusion, transplacentally, and/or through sexual transmission.

**[0005]** In late 2015, a significant increase in fetal abnormalities (*e.g.,* microcephaly) and Guillain-Barre syndrome (GBS) in areas of widespread Zika virus infection raised alarm that Zika virus might be much more virulent than originally thought, prompting the World Health Organization (WHO) to declare a Public Health Emergency of International Concern (PHEIC) (Heymann *et al.* (2016) Lancet 387(10020): 719-21). While Zika virus poses a substantial public health threat, no FDA-approved vaccine or treatment currently exists, and the only preventative measures for controlling Zika virus involve managing mosquito populations.

**[0006]** In recent efforts to characterize a recombinant Zika virus for the development of a potential vaccine, a non-human cell adapted Zika virus was identified that harbors a mutation in the viral Envelope protein at position 330 (Weger-Lucarelli *et al.* 2017. Journal of Virology). The authors of this study found that full-length infectious cDNA clones of Zika virus strain PRVABC59 were genetically unstable when amplified during cloning, and opted to split the viral genome to address the observed instability, developing and applying a two plasmid system. However, a two plasmid system for the development of a Zika vaccine is less desirable.

## BRIEF SUMMARY

**[0007]** Thus, there is a need to develop vaccines and immunogenic compositions for treating and/or preventing Zika virus infection that utilize a genetically stable Zika virus. One option for the development of a vaccine is to inactivate a whole virus and use this inactivated whole virus for the vaccination of subjects. However, during the development of an inactivated viral vaccine, a key safety assurance is to be certain that no infectious virus remains in the drug product or drug substance. Developing an effective inactivation process and sensitive analytics to measure and determine if infectious virions remain is a key safety example for the development of a purified inactivated virus derived from any wild-type virus, but certainly with a pathogenic/encephalitic virus that could cause fetal abnormalities. Further, formaldehyde which may be used for inactivating a virus is known to be genotoxic and carcinogenic so that it is important to monitor residual levels of formaldehyde in drug substances and drug products and regulatory authorities require manufacturers using formaldehyde as inactivating agent to determine the residual formaldehyde

content in the drug product. Hence, there is a need for a sensitive method for detecting residual formaldehyde in a drug product or pharmaceutical composition containing an inactivated virus such as an inactivated Zika virus.

**[0008]** The present disclosure is based, at least in part, on the surprising finding that Zika virus can efficiently be inactivated with a low concentration of formaldehyde which is applied to the virus for a relatively short time at room temperature. Additionally, an assay was developed which allows to determine with a high sensitivity whether infectious virions are still present after inactivation. Finally, a method was developed which allows to detect low levels of residual formaldehyde in the final drug product or pharmaceutical composition.

**[0009]** In one aspect, the present disclosure provides a pharmaceutical composition comprising a Zika virus inactivated with formaldehyde, the pharmaceutical composition having a residual formaldehyde content of less than 50 μg/mL and less than 1.0 TCID50 of residual replicating Zika virus. In another aspect, the present disclosure provides a use of the pharmaceutical composition as described herein in the manufacture of a medicament for treating or preventing Zika virus infection in a subject in need thereof, and/or inducing an immune response, such as a protective immune response, against Zika virus in a subject in need thereof.

**[0009a]** Accordingly, certain examples of the present disclosure relate to a method for inactivating a Zika virus preparation comprising:
(a) isolating the Zika virus preparation from one or more cells cultured *in vitro*, wherein the cells are used to produce the Zika virus preparation, wherein isolating the Zika virus preparation comprises one or more steps selected from:
(i) depth filtration,
(ii) buffer exchange and/or dilution;
(iii) ion exchange chromatography; and
(b) treating the Zika virus preparation with formaldehyde, wherein the numerical result of the multiplication of the formaldehyde concentration as measured in % (w/v) with the period of incubation with formaldehyde as measured in days is 0.025 to 0.5.

**[0010]** In some examples, the cells are non-human cells or Vero cells.

**[0011]** In some examples, the Zika virus preparation is obtained from an inoculum containing a heterogeneous population of Zika viruses.

**[0012]** In some examples, the Zika virus preparation is obtained from a clinical isolate.

**[0013]** In some examples, the Zika virus preparation is obtained from a Zika virus clonal isolate. The Zika virus clonal isolate may be obtained by plaque purification. Prior to plaque purification a plurality of cells may be inoculated with an inoculum containing a heterogenous population of Zika viruses.

**[0014]** Some examples of the present disclosure relate to a method for inactivating a Zika virus preparation comprising:
(a) obtaining a Zika virus preparation from a clinical isolate; and
(b) treating the Zika virus preparation with formaldehyde, wherein the numerical result of the multiplication of the formaldehyde concentration as measured in % (w/v) with the period of incubation with formaldehyde as measured in days is 0.025 to 0.5.

**[0015]** Some examples of the present disclosure relate to a method for inactivating a Zika virus preparation comprising:
(a) obtaining a Zika virus preparation from an inoculum containing a heterogeneous population of Zika viruses; and
(b) treating the Zika virus preparation with formaldehyde, wherein the numerical result of the multiplication of the formaldehyde concentration as measured in % (w/v) with the period of incubation with formaldehyde as measured in days is 0.025 to 0.5.

**[0016]** In some examples, the Zika virus preparation is treated with formaldehyde at a concentration of 0.005 % (w/v) to 0.02 % (w/v).

**[0017]** In some examples, the Zika virus preparation is treated for eight to twelve days or for ten days.

**[0018]** In some examples, the Zika virus preparation is treated at a temperature of 15°C to 30°C or of 22°C.

**[0019]** The method may further comprise a step (c) of determining the completeness of inactivation.

**[0020]** In some examples, step (c) comprises:

(i) inoculating cultured insect cells with a Zika virus preparation treated according to step (b) and incubating the insect cells for a first period of time, thereby producing an insect cell supernatant;
(ii) inoculating cultured mammalian cells with the insect cell supernatant produced in (i) and incubating the mammalian cells for a second period of time; and
(iii) determining whether the Zika virus preparation contains a residual replicating virus that produces a cytopathic effect on the mammalian cells.

**[0021]** In some examples, the insect cells are selected from CCL-125 cells, Aag-2 cells, RML-12 cells, C6/36 cells, C7-10 cells, AP-61 cells, A.t. GRIP-1 cells, A.t. GRIP-2 cells, A.t. GRIP-3 cells, UM-AVE1 cells, Mos.55 cells, Sua1B cells, 4a-3B cells, Mos.42 cells, MSQ43 cells, LSB-AA695BB cells, NIID-CTR cells and TRA-171 cells, such as C6/36 cells.

**[0022]** In some examples, the first period of time is 3 to 7 days.

**[0023]** In some examples, the mammalian cells are selected from VERO cells, LLC-MK2 cells, MDBK cells, MDCK cells, ATCC CCL34 MDCK (NBL2) cells, MDCK 33016 (deposit number DSM ACC 2219 as described in WO97/37001) cells, BHK21-F cells, HKCC cells, and Chinese hamster ovary cells (CHO cells), such as VERO cells.

**[0024]** In some examples, the second period of time is 3 to 14 days.

**[0025]** The method may further comprise a step (d) of neutralizing the formaldehyde-treated Zika virus preparation with sodium metabisulfite, such as neutralizing the formaldehyde-treated Zika virus preparation at least five, at least seven, at least nine, at least 11, or at least 14 days after formaldehyde treatment.

**[0026]** The method may further comprise a step (e) of preparing a pharmaceutical composition comprising the inactivated Zika virus preparation.

**[0027]** In some examples, the Zika virus preparation is mixed with an adjuvant. The adjuvant may be selected from the group consisting of aluminum salts, toll-like receptor (TLR) agonists, monophosphoryl lipid A (MLA), synthetic lipid A, lipid A mimetics or analogs, MLA derivatives, cytokines, saponins, muramyl dipeptide (MDP) derivatives, CpG oligos, lipopolysaccharide (LPS) of gram-negative bacteria, polyphosphazenes, emulsions, virosomes, cochleates, poly(lactide-co-glycolides) (PLG) microparticles, poloxamer particles, microparticles, liposomes, Complete Freund's Adjuvant (CFA), and Incomplete Freund's Adjuvant (IFA).

**[0028]** In some examples, the adjuvant is an aluminum salt, such as aluminum phosphate, aluminum hydroxide, potassium aluminum sulfate, or Alhydrogel 85.

**[0029]** In some examples, at least 75%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% of one or more antigens in the Zika virus preparation are adsorbed to the adjuvant.

**[0030]** In some examples, the Zika virus comprises a mutation at position 98 of SEQ ID NO: 1 or at a position corresponding to position 98 of SEQ ID NO: 1, such as a Trp98Gly mutation in SEQ ID NO: 1.

**[0031]** In some examples, the Zika virus does not comprise a mutation in the envelope protein (E). In some examples, the sequence encoding the envelope protein is the same as the corresponding sequence in SEQ ID NO: 2.

**[0032]** Some examples of the present disclosure also relate to a pharmaceutical composition comprising an inactivated Zika virus obtainable by any of the methods described herein.

**[0033]** Some examples of the present disclosure also relate to a pharmaceutical composition comprising an inactivated Zika virus and having a residual formaldehyde content of less than 50 µg/ml. In some examples, the pharmaceutical composition is obtainable by the method of any one of claims 1 to 28.

**[0034]** Some examples of the present disclosure also relate to a method for determining the completeness of inactivation of an arbovirus preparation, comprising the steps of:
(i) inoculating cultured insect cells with an arbovirus preparation which was subjected to an inactivation step and incubating the insect cells for a first period of time, thereby producing an insect cell supernatant;
(ii) inoculating cultured mammalian cells with the insect cell supernatant produced in (i) and incubating the mammalian cells for a second period of time; and
(iii) determining whether the arbovirus preparation contains a residual replicating virus that produces a cytopathic effect on the mammalian cells.

**[0035]** In some examples, the arbovirus is a flavivirus or an alphavirus. In some examples, the arbovirus is a Zika virus, a West Nile virus, a Yellow Fever virus, a Japanese Encephalitis virus, a

tick borne-encephalitis virus. a dengue virus, a St. Louis Encephalitis virus, a Chikungunya virus, a O'nyong'nyong virus or a Mayarovirus.

**[0036]** In some examples, the arbovirus preparation was subjected to a treatment with detergent, formalin, hydrogen peroxide, beta-propiolactone (BPL), binary ethylamine (BEI), acetyl ethyleneimine, methylene blue, or psoralen.

**[0037]** In some examples, the insect cells are selected from CCL-125 cells, Aag-2 cells, RML-12 cells, C6/36 cells, C7-10 cells, AP-61 cells, A.t. GRIP-1 cells, A.t. GRIP-2 cells, A.t. GRIP-3 cells, UM-AVE1 cells, Mos.55 cells, Sua1B cells, 4a-3B cells, Mos.42 cells, MSQ43 cells, LSB-AA695BB cells, NIID-CTR cells and TRA-171 cells, such as C6/36 cells.

**[0038]** In some examples, the first period of time is 3 to 7 days.

**[0039]** In some examples, the mammalian cells are selected from VERO cells, LLC-MK2 cells, MDBK cells, MDCK cells, ATCC CCL34 MDCK (NBL2) cells, MDCK 33016 (deposit number DSM ACC 2219 as described in WO97/37001) cells, BHK21-F cells, HKCC cells, and Chinese hamster ovary cells (CHO cells), such as VERO cells.

**[0040]** In some examples, the second period of time is 3 to 14 days.

**[0041]** In some examples, the method is capable of detecting less than 1.0 $TCID_{50}$ of the arbovirus.

**[0042]** Some examples of the present disclosure also relate to a method for determining the residual formaldehyde content in a pharmaceutical composition comprising an inactivated virus, comprising the steps of:
(a) providing a composition comprising a virus which has been treated with formaldehyde;
(b) mixing the composition of (a) with phosphoric acid and 2,4-dinitrophenylhydrazine (DNPH), thereby providing a mixture;
(c) incubating the mixture of (b) under suitable conditions; and
(d) analyzing the mixture for the presence of residual formaldehyde.

**[0043]** In some examples, the composition of (a) contains an adjuvant which may be aluminum hydroxide. In some examples, the composition of (a) contains 0.1 mg/ml to 1.0 mg/ml aluminum hydroxide as adjuvant.

**[0044]** In some examples, step (b) comprises mixing 50 parts of the composition of (a) with 1 part of 15 to 25% (v/v) phosphoric acid and 2.5 parts of 0.9 to 1.1 mg/ml DNPH.

**[0045]** In some examples, the the mixture of the composition of (a) with phosphoric acid and 2,4-dinitrophenylhydrazine (DNPH) is incubated at room temperature. In some examples, the mixture of the composition of (a) with phosphoric acid and 2,4-dinitrophenylhydrazine (DNPH) is incubated for 10 to 30 minutes.

**[0046]** In some examples, the the mixture of the composition of (a) with phosphoric acid and 2,4-dinitrophenylhydrazine (DNPH) is analyzed by HPLC which may be reversed-phase HPLC. In some examples, a mixture of water and acetonitrile (1:1, v/v) is used as a mobile phase in HPLC.

**[0047]** In some examples, the virus is an inactivated Zika virus. In some examples, the inactivated Zika virus has been treated with 0.01% (w/v) formaldehyde for 10 days at 22°C. In some examples, the Zika virus comprises a mutation at position 98 of SEQ ID NO: 1 or at a position corresponding to position 98 of SEQ ID NO: 1, such as a Trp98Gly mutation in SEQ ID NO: 1.

## BRIEF DESCRIPTION OF THE DRAWINGS

**[0048]** **FIG. 1** shows bright field microscopy images of Vero cell monolayers mock infected (top) or infected with ZIKAV strain PRVABC59 (bottom).

**[0049]** **FIG. 2** shows growth kinetics of ZIKAV PRVABC59 P1 on Vero cell monolayers, as determined by $TCID_{50}$.

**[0050]** **FIG. 3** shows potency assay testing ($TCID_{50}$) of Zika virus PRVABC59 P5 clones a-f.

**[0051]** **FIG. 4** shows bright-field microscopy images depicting the cytopathic effect (CPE) of growth of Zika virus PRVABC59 P6 clones a-f on Vero cell monolayers.

**[0052]** **FIG. 5** shows potency assay testing ($TCID_{50}$) of Zika virus PRVABC59 P6 clones a-f

**[0053]** **FIG. 6** shows an amino acid sequence alignment comparing the envelope glycoprotein sequence of Zika virus near residue 330 from Zika virus strains PRVABC59 P6e (SEQ ID NO: 8) and PRVABC59 (SEQ ID NO: 9) with several other flaviviruses (WNV (SEQ ID NO: 10); JEV (SEQ ID NO: 11); SLEV (SEQ ID NO: 12); YFV (SEQ ID NO: 13); DENV 1 16007 (SEQ ID NO:

14); DENV 2 16681 (SEQ ID NO: 15); DENV 3 16562 (SEQ IDNO: 16); and DENV 4 1036 (SEQ ID NO: 17)).

CLAIMS

1. A pharmaceutical composition comprising a Zika virus inactivated with formaldehyde, the pharmaceutical composition having a residual formaldehyde content of less than 50 µg/mL and less than 1.0 TCID50 of residual replicating Zika virus.

2. The pharmaceutical composition according to claim 1, wherein the residual formaldehyde is determined by a method for determining the residual formaldehyde content in a pharmaceutical composition comprising an inactivated virus, the method comprising the steps of:
(a) providing a composition comprising a virus which has been treated with formaldehyde;
(b) mixing the composition of (a) with phosphoric acid and 2,4-dinitrophenylhydrazine (DNPH), thereby providing a mixture;
(c) incubating the mixture of (b) under suitable conditions; and
(d) analyzing the mixture for the presence of residual formaldehyde.

3. The pharmaceutical composition of claim 1, wherein residual replicating Zika virus is determined by a method for determining the completeness of inactivation of a Zika virus preparation, comprising the steps of:
(i) inoculating cultured insect cells, such as C6/36 cells with a Zika virus preparation which was subjected to an inactivation step and incubating the insect cells for a first period of time, preferably for 3 to 7 days, thereby producing an insect cell supernatant;
(ii) inoculating cultured mammalian cells, such as Vero cells, with the insect cell supernatant produced in (i) and incubating the mammalian cells for a second period of time, preferably for 3 to 14 days; and
(iii) determining whether the Zika virus preparation contains a residual replicating virus that produces a cytopathic effect on the mammalian cells.

4. The pharmaceutical composition of any one of claims 1 to 3, further comprising an adjuvant, preferably wherein the adjuvant is an aluminum salt, such as aluminum hydroxide.

5. The pharmaceutical composition of claim 4, wherein at least 75%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% of the Zika virus in the pharmaceutical composition are adsorbed to the adjuvant.

6. The pharmaceutical composition of any one of claims 1 to 5, wherein the composition is made by a process comprising a step of removing residual unreacted formaldehyde by:
-neutralizing the formaldehyde-treated Zika virus preparation with sodium metabisulfite,
-dialysis,
-buffer exchange, and/or
-tangential flow filtration (TFF).

7. The pharmaceutical composition of claim 6, wherein the composition is made by a process comprising a step of removing residual unreacted formaldehyde by neutralizing the formaldehyde-treated Zika virus preparation with from about 1 mM to about 10 mM sodium metabisulfite.

8. The pharmaceutical composition of any one of claims 1 to 7, wherein the composition is made by a process comprising a step of inactivating the Zika virus with formaldehyde, wherein the numerical result of the multiplication of the formaldehyde concentration as measured in % (w/v) with the period of incubation with formaldehyde as measured in days is 0.025 to 0.5, optionally wherein the Zika virus is inactivated at a temperature of 15 °C to 30 °C.

9. The pharmaceutical composition of claim 8, wherein the composition is made by a process comprising a step of inactivating the Zika virus with 0.005 to 0.02% (w/v) formaldehyde for eight to twelve days at a temperature of 15 °C to 30 °C, preferably with 0.01% (w/v) formaldehyde for ten days at a temperature of 22 °C.

10. The pharmaceutical composition of any one of claims 1 to 9, having a residual formaldehyde content of less than 45 µg/mL, less than 40 µg/mL, less than 35 µg/mL, less than 30 µg/mL, less than 25 µg/mL, less than 20 µg/mL, less than 15 µg/mL, or

less than 10 µg/mL, less than 5 µg/mL, less than 4 µg/mL, less than 3 µg/mL, less than 2 µg/mL, less than 1.5 µg/mL, less than 1 µg/mL, or less than 0.5 µg/mL.

11. The pharmaceutical composition of any one of claims 1 to 10, having less than 0.8 TCID50, less than 0.5 TCID50, less than 0.2 TCID50, or less than 0.1 TCID50 of residual replicating Zika virus.

12. The pharmaceutical composition of any one of claims 1 to 11, wherein the inactivated Zika virus is purified and the main peak of the purified inactivated Zika virus when analyzed by size exclusion chromatography is more than 85% or more than 90% or more than 95% of the total area under the curve in the size exclusion chromatography.

13. The pharmaceutical composition of any one of claims 1 to 12, comprising from about 0.1 µg to about 100 µg of inactivated Zika virus.

14. The pharmaceutical composition of any one of claims 1 to 13, further comprising a pharmaceutically acceptable excipient such as sucrose, dextrose, glycerol, ethanol, saline, water, or any combination thereof.

15. The pharmaceutical composition of any one of claims 1 to 14, further comprising one or more pharmaceutically acceptable buffers such as Tris buffer, phosphate buffer, borate buffer, succinate buffer, histidine buffer, or citrate buffer, optionally wherein the pharmaceutical composition comprises the one or more pharmaceutically acceptable buffers in the range of 5 to 20 mM and/or wherein the pH of the pharmaceutical composition is between 5.0 and 8.5.

16. The pharmaceutical composition of any one of claims 1 to 15, further comprising a physiological salt such as sodium chloride, potassium chloride, magnesium chloride or calcium chloride, optionally at a concentration between 1 and 20 mg/mL.

17. Use of the pharmaceutical composition of any one of claims 1 to 16 in the manufacture of a medicament

for treating or preventing Zika virus infection in a subject in need thereof, and/or

inducing an immune response, such as a protective immune response, against Zika virus in a subject in need thereof.

18. The use according to claim 17, wherein the subject is a human, optionally wherein the subject is pregnant or intends to become pregnant.