[0008a] In one aspect, the present disclosure relates 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, wherein the insect cells can be infected by the arbovirus, 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), wherein the mammalian cells can be infected by the arbovirus, 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.

[0008b] In another aspect, the present disclosure relates 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;

(b) treating the Zika virus preparation with 0.005% to 0.02% w/v of formaldehyde, preferably with 0.01% w/v formaldehyde;

and further removing residual unreacted formaldehyde and determining the completeness of inactivation according to the method as described herein, wherein the arbovirus preparation is the Zika virus preparation treated according to step (b).

[0008c] In yet another aspect, the present disclosure relates to a pharmaceutical composition comprising an inactivated Zika virus obtained by the method as described herein. In a further aspect, the present disclosure relates to a pharmaceutical composition comprising an inactivated Zika virus preparation, wherein the inactivated Zika virus preparation comprises less than 1.0 TCID50 of residual replicating virus as determined by the method as described herein.

[0009] 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 virus preparation; and

(b) treating the Zika virus preparation with 0.005% to 0.02% w/v of formaldehyde.

[0010] In some examples, the cells are non-human cells. In some examples, the Zika virus preparation is treated with 0.01% formaldehyde. In some examples, the Zika virus preparation is treated for eight to twelve days, such as for ten days. In some examples, the Zika virus preparation is treated at a temperature of 15°C to 30°C, such as a temperature of 22°C.

[0011] The method may further comprise a step (c) of determining the completeness of inactivation. In some examples, step (c) comprises:

(i) inoculating cultured insect cells with a Zika virus preparation treated with 0.005% to 0.02% w/v of formaldehyde 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.

[0012] 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.

[0013] In some examples, the first period of time is 3 to 7 days.

[0014] 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.

[0015] In some examples, the second period of time is 3 to 14 days.

[0016] 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.

[0017] The method may further comprise a step (e) of preparing a pharmaceutical composition comprising the inactivated Zika virus.

[0018] In some examples, the treated 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).

[0019] In some examples, the adjuvant is an aluminum salt, such as aluminum phosphate, aluminum hydroxide, potassium aluminum sulfate, and Alhydrogel 85.

[0020] 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 treated virus preparation are adsorbed to the adjuvant.

[0021] 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.

[0022] 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.

[0023] Some examples of the present disclosure relate to a pharmaceutical composition comprising an inactivated Zika virus obtainable by any of the methods disclosed herein.

[0024] Some examples of the present disclosure relate to a pharmaceutical composition comprising an inactivated Zika virus and having a residual formalin content of less than 0.5 μg/ml. In some examples, the pharmaceutical composition is obtainable by any of the methods disclosed herein.

[0025] Some examples of the present disclosure relate to a method for determining the residual formalin content in a pharmaceutical composition comprising an inactivated virus, comprising the steps of:
(a) providing a pharmaceutical composition comprising a virus which has been treated with formaldehyde;
(b) mixing the pharmaceutical composition of (a) with phosphoric acid and 2,4-dinitrophenyl-hydrazine (DNPH), thereby providing a mixture;
(c) incubating the mixture of (b) under suitable conditions; and
(d) analyzing the mixture for the presence of residual formalin.

[0026] In some examples, the pharmaceutical composition contains an adjuvant. In some examples, the pharmaceutical composition contains aluminum hydroxide as adjuvant. In some examples, the pharmaceutical composition contains 0.1 mg/ml to 1.0 mg/ml aluminum hydroxide as adjuvant. In some examples, the pharmaceutical composition contains 0.4 mg/ml aluminum hydroxide as adjuvant.

[0027] In some examples, a volume of 1 ml of the pharmaceutical composition of (a) is mixed with 20 µl of 15 to 25% (v/v) phosphoric acid and 50 µl of 0.9 to 1.1 mg/ml DNPH. In some examples, a volume of 1 ml of the pharmaceutical composition of (a) is mixed with 20 µl of 20% (v/v) phosphoric acid and 50 µl of 1.0 mg/ml DNPH.

[0028] In some examples, the mixture of the pharmaceutical composition of (a) with phosphoric acid and 2,4-dinitrophenylhydrazine (DNPH) is incubated at room temperature. In some examples, the mixture of the pharmaceutical composition of (a) with phosphoric acid and 2,4-dinitrophenylhydrazine (DNPH) is incubated for 10 to 30 minutes. In some examples, the mixture of the pharmaceutical composition of (a) with phosphoric acid and 2,4-dinitrophenylhydrazine (DNPH) is incubated at room temperature for 20 minutes.

[0029] In some examples, the mixture of the pharmaceutical composition of (a) with phosphoric acid and 2,4-dinitrophenylhydrazine (DNPH) is analyzed by HPLC. In some examples, the HPLC is reversed-phase HPLC. In some examples, a mixture of water and acetonitrile (1:1, v/v) is used as a mobile phase in HPLC. In some examples, the detection wavelength is 360 nm.

[0030] 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.

[0031] Some examples of the present disclosure 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.

[0032] 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, tick borne encephalitis virus, a dengue virus, a St. Louis Encephalitis virus, a Chikungunya virus, a O'nyong'nyong virus or a Mayarovirus.

[0033] In some examples, the arbovirus preparation was subjected to an inactivation step with detergent, formalin, hydrogen peroxide, beta-propiolactone (BPL), binary ethylamine (BEI), acetyl ethyleneimine, methylene blue, or psoralen.

[0034] 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.

[0035] In some examples, the first period of time is 3 to 7 days.

[0036] 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.

[0037] In some examples, the second period of time is 3 to 14 days.

[0038] In some examples, the method is capable of detecting less than 1.0 TCID50 of the arbovirus.

BRIEF DESCRIPTION OF THE DRAWINGS

[0039] **FIG. 1** shows bright field microscopy images of Vero cell monolayers mock infected (top) or infected with ZIKAV strain PRVABC59 (bottom).

[0040] **FIG. 2** shows growth kinetics of ZIKAV PRVABC59 P1 on Vero cell monolayers, as determined by $TCID_{50}$.

[0041] **FIG. 3** shows potency assay testing ($TCID_{50}$) of Zika virus PRVABC59 P5 clones a-f.

[0042] **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.

[0043] **FIG. 5** shows potency assay testing ($TCID_{50}$) of Zika virus PRVABC59 P6 clones a-f

[0044] **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)).

[0045] **FIG. 7** shows an amino acid sequence alignment comparing the NS1 protein sequence of Zika virus near residue 98 from Zika virus strains PRVABC59 P6e (SEQ ID NO: 18) and PRVABC59 (SEQ ID NO: 19) with several other flaviviruses (WNV (SEQ ID NO: 20); JEV (SEQ ID NO: 21); SLEV (SEQ ID NO: 22); YFV (SEQ ID NO: 23); DENV 1 16007 (SEQ ID NO: 24); DENV 2 16681 (SEQ ID NO: 25); DENV 3 16562 (SEQ IDNO: 26); and DENV 4 1036 (SEQ ID NO: 27)).

[0046] **FIG. 8** shows the plaque phenotype of ZIKAV PRVABC59 P6 virus clones a-f compared to ZIKAV PRVABC59 P1 virus.

CLAIMS

1. 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, wherein the insect cells can be infected by the arbovirus, 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), wherein the mammalian cells can be infected by the arbovirus, 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.

2. The method of claim 1, wherein 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.

3. The method of any one of claims 1 or 2, wherein the first period of time is 3 to 7 days.

4. The method of any one of claims 1 to 3, wherein 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.

5. The method of any one of claims 1 to 4, wherein the second period of time is 3 to 14 days.

6. The method of any one of claims 1 to 5, wherein the arbovirus preparation was subjected to a treatment with detergent, formaldehyde, hydrogen peroxide, beta-propiolactone (BPL), binary ethylamine (BEI), acetyl ethyleneimine, methylene blue, or psoralen.

7. The method of any one of claims 1 to 6, wherein the arbovirus is a flavivirus or an alphavirus, preferably wherein 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.

8. The method according to any one of claims 1 to 7 for determining the completeness of inactivation of a Zika virus preparation, comprising the steps of:

(i) inoculating C6/36 cells with a Zika virus preparation which was subjected to an inactivation step and incubating the C6/36 cells for a first period of time, such as for 3 to 7 days, thereby producing a C6/36 cell supernatant;

(ii) inoculating Vero cells with the C6/36 cell supernatant produced in (i) and incubating the Vero cells for a second period of time, such as 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 Vero cells.

9. The method of any one of claims 1 to 8, wherein the method is capable of detecting less than 1.0 $TCID_{50}$ of the arbovirus.

10. 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;

(b) treating the Zika virus preparation with 0.005% to 0.02% w/v of formaldehyde, preferably with 0.01% w/v formaldehyde;

and

further removing residual unreacted formaldehyde and determining the completeness of inactivation according to the method of any one of claims 1 to 9, wherein the arbovirus preparation is the Zika virus preparation treated according to step (b).

11. The method of claim 10, wherein the cells in step (a) are non-human cells.

12. The method of any one of claims 10 or 11, wherein the Zika virus preparation is treated for eight to twelve days, preferably wherein the Zika virus preparation is treated for ten days.

13. The method of any one of claims 10 to 12, wherein the Zika virus preparation is treated at a temperature of 15°C to 30°C, preferably at a temperature of 22°C.

14. The method of any one of claims 10 to 13, wherein residual unreacted formaldehyde is removed by

neutralizing the formaldehyde-treated Zika virus preparation with sodium metabisulfite,

dialysis,

buffer exchange, or

tangential flow filtration (TFF).

15. The method of any one of claims 10 to 14, further comprising a step (c) of preparing a pharmaceutical composition comprising the inactivated Zika virus preparation.

16. The method of claim 15, wherein the Zika virus preparation is mixed with an adjuvant, preferably wherein the adjuvant is 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), more preferably wherein the adjuvant is an aluminum salt, such as aluminum hydroxide, aluminum phosphate, potassium aluminum sulfate, and Alhydrogel 85.

17. The method of claim 16, 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 one or more antigens in the Zika virus preparation are adsorbed to the adjuvant.

18. A pharmaceutical composition comprising an inactivated Zika virus obtained by the method of any one of claims 10 to 17.

19. The pharmaceutical composition of claim 18, having a residual formalin content of less than 0.5 µg/ml.

20. A pharmaceutical composition comprising an inactivated Zika virus preparation, wherein the inactivated Zika virus preparation comprises less than 1.0 TCID50 of residual replicating virus as determined by the method according to any one of claims 1 to 9.