(2009) *BMC Infect Dis* 9: 3; and Wang, SM et al., (1999) *Clin Infect Dis* 29: 184–190). Preventive and control measures during EV71 outbreaks are limited to surveillance, closure of educational and childcare facilities, and isolation of patients.

[0009] For production of potential vaccines, viral vaccines are usually produced by anchorage-dependent cell lines (e.g. VERO cells). At industrial scale, these cells are either cultivated in static mode on multi-plate systems (e.g., Cell Factories, Cell Cube, etc.), on roller bottles, or on microcarriers (porous or non-porous) in suspension in bioreactors. Multi-plate systems are bulky and require significant handling operations, whereas microcarrier cultures require numerous operations (sterilization and hydration of carriers, etc.) and many steps from precultures to final process with complex operations (i.e. bead-to-bead transfers).

[0010] Like many other enteric viruses, Enterovirus A (e.g., EV71 virus) has been adapted to grow on cell lines (e.g., Vero cells) that produce CPE with complete cell lysis, releasing the virus into the cell culture fluid. Enterovirus A (e.g., EV71 virus) virus culture is more challenging than other viruses due to lower quantum of virus production per cell and rapid cytolysis, resulting in low yields per culture run. As cell lysis occurs very rapidly, the production of large quantities of viral particles is a massive task requiring several large cell culture devices of conventional systems like hundreds of roller bottles or cell factories or several liters of large scale microcarrier culture tanks, thus requiring prolonged preculture steps as well as complex multiple operations. The current regulatory environment demands avoiding reusable culture methods as well as multiple steps of asceptic operations and transfers riddled with the risk of contaminations. However, most of the currently-available single-use reactors are not well-adapted to cultivate animal cells immobilized on micro carriers.

SUMMARY

[0011] Thus, there is a need to develop methods for Enterovirus A production, *e.g.,* methods that allow for viral production on an industrial scale. As disclosed herein, the methods of the present disclosure, *inter alia,* use a fixed bed culture system to enable enhanced viral production with greater cost efficiency and streamlined processing. Advantageously, these methods may be used, for example, for large- or industrial-scale production of an Enterovirus A, which is useful in the manufacture of vaccines and/or immunogenic compositions.

[0012] Accordingly, in one aspect, the present disclosure refers to a method for producing an Enterovirus A virus, comprising (a) culturing an adherent cell in a fixed bed comprising a

macrocarrier, where the cell is cultured in a first cell culture medium; (b) inoculating the cell with the Enterovirus A virus under conditions in which the Enterovirus A virus infects the cell; (c) culturing the infected cell in the fixed bed in a second cell culture medium under conditions in which the infected cell produces the Enterovirus A virus; and (d) harvesting the Enterovirus A virus produced by the cell, wherein (i) the cell is inoculated with the Enterovirus A virus at a multiplicity of infection (MOI) of between 0.025 and 0.0009, optionally 0.001, and/or (ii) between 200,000 cells/cm^2 and 350,000 cells/cm^2 are inoculated in step (b). In some examples that may be combined with any of the above examples, the cell is a mammalian cell. In some examples that may be combined with any of the above examples, the cell is a Vero cell. In some examples that may be combined with any of the above examples, the Vero cell line is selected from a WHO Vero 10-87, ATCC CCL-81, Vero 76 (ATCC Accession No. CRL-1587), and Vero C1008 (ATCC Accession No. CRL-1586). In some examples that may be combined with any of the above examples, the Enterovirus A virus is selected from EV71, CA6, and CA16. In some examples that may be combined with any of the above examples, the Enterovirus A virus contains at least one non-human cell adaptation mutation. In some examples that may be combined with any of the above examples, the Enterovirus A virus is EV71, and the at least one non-human cell adaptation mutation includes a VP1 polypeptide mutation. In some examples that may be combined with any of the above examples, the Enterovirus A virus is CA6, and the at least one non-human cell adaptation mutation includes a mutation selected from a VP1 polypeptide mutation, a VP2 polypeptide mutation, a VP3 polypeptide mutation, and a 5' untranslated region (UTR) mutation. In some examples that may be combined with any of the above examples, the Enterovirus A virus is CA16, and the at least one non-human cell adaptation mutation includes a mutation selected from a 2A polypeptide mutation, a VP1 polypeptide mutation, a VP2 polypeptide mutation, and a 5' untranslated region (UTR) mutation. In some examples that may be combined with any of the above examples, between about 4,000 cells/cm^2 and about 16,000 cells/cm^2 are cultured in the step of culturing an adherent cell in a fixed bed containing a macrocarrier, where the cell is cultured in a first cell culture medium. In some examples that may be combined with any of the above examples, about 5,000 cells/cm^2 are cultured in the step of culturing an adherent cell in a fixed bed containing a macrocarrier, where the cell is cultured in a first cell culture medium. In some examples that may be combined with any of the above examples, between about 100,000 cells/cm^2 and about 800,000 cells/cm^2 are inoculated in the step of inoculating the cell with the Enterovirus A virus under conditions in which the Enterovirus A virus infects the cell. In some examples that may be combined with any of the above examples, between about 200,000 cells/cm^2 and about 350,000 cells/cm^2 are inoculated in the step of

inoculating the cell with the Enterovirus A virus under conditions in which the Enterovirus A virus infects the cell. In some examples that may be combined with any of the above examples, the first cell culture medium contains serum. In some examples that may be combined with any of the above examples, the first cell culture medium contains less than 10% fetal bovine serum, and the cell is not adapted to serum free medium. In some examples that may be combined with any of the above examples, the first cell culture medium contains 5% fetal bovine serum. In some examples that may be combined with any of the above examples, the first cell culture medium and the second cell culture medium are different. In some examples that may be combined with any of the above examples, the method further includes, between the steps of culturing an adherent cell in a fixed bed containing a macrocarrier, where the cell is cultured in a first cell culture medium and inoculating the cell with the Enterovirus A virus under conditions in which the Enterovirus A virus infects the cell, removing the first cell culture medium and rinsing the cell with the second culture medium. In some examples that may be combined with any of the above examples, the second cell culture medium is a serum-free medium. In some examples that may be combined with any of the above examples, the cell is inoculated with the Enterovirus A virus at an MOI of between about 0.025 and about 0.0009. In some examples that may be combined with any of the above examples, the cell is inoculated with the Enterovirus A virus at an MOI of about 0.001. In some examples that may be combined with any of the above examples, the cell is cultured during the steps of culturing an adherent cell in a fixed bed containing a macrocarrier, where the cell is cultured in a first cell culture medium, inoculating the cell with the Enterovirus A virus under conditions in which the Enterovirus A virus infects the cell, and culturing the infected cell in the fixed bed in a second cell culture medium under conditions in which the infected cell produces the Enterovirus A virus, at a volume/surface ratio of about 0.3 mL/cm^2. In some examples that may be combined with any of the above examples, the lactate concentration in the first cell culture medium during the steps of culturing an adherent cell in a fixed bed containing a macrocarrier, where the cell is cultured in a first cell culture medium and/or inoculating the cell with the Enterovirus A virus under conditions in which the Enterovirus A virus infects the cell does not exceed about 25 mM. In some examples that may be combined with any of the above examples, the lactate concentration in the second cell culture medium during the step of culturing the infected cell in the fixed bed in a second cell culture medium under conditions in which the infected cell produces the Enterovirus A virus does not exceed about 25 mM. In some examples that may be

combined with any of the above examples, the density of oxygen (DO) in the first cell culture medium during the steps of culturing an adherent cell in a fixed bed containing a macrocarrier, where the cell is cultured in a first cell culture medium and/or inoculating the cell with the Enterovirus A virus under conditions in which the Enterovirus A virus infects the cell is maintained above about 50%. In some examples that may be combined with any of the above examples, the density of oxygen (DO) in the second cell culture medium during the step of culturing the infected cell in the fixed bed in a second cell culture medium under conditions in which the infected cell produces the Enterovirus A virus is maintained above about 50%. In some examples that may be combined with any of the above examples, the fixed bed has a bed height of about 2 cm. In some examples that may be combined with any of the above examples, the fixed bed has a bed height of about 10 cm. In some examples that may be combined with any of the above examples, the macrocarrier is a microfiber matrix. In some examples that may be combined with any of the above examples, the macrocarrier has a porosity between about 60% and 99%. In some examples that may be combined with any of the above examples, the porosity is between about 80% and about 90%. In some examples that may be combined with any of the above examples, the macrocarrier has a surface area accessible to the cell of between about 150 cm^2/cm^3 and about 1000 cm^2/cm^3. In some examples that may be combined with any of the above examples, at least 5.0 x 10^7 TCID50/mL of the Enterovirus A virus is harvested in the step of harvesting the Enterovirus A virus produced by the cell. In some examples that may be combined with any of the above examples, the methods further include inactivating the Enterovirus A with one or more of beta-propiolactone (BPL), formalin, or binary ethylenimine (BEI). In some examples that may be combined with any of the above examples, the Enterovirus A virus is an attenuated virus.

[0013] Other examples of the present disclosure relate to an Enterovirus A virus produced by the method of any of the above examples. In some examples that may be combined with any of the above examples, the virus contains one or more antigens. In some examples that may be combined with any of the above examples, the virus has been inactivated with one or more of beta-propiolactone (BPL), formalin, or binary ethylenimine (BEI).

[0013a] Other examples of the present disclosure relate to a composition comprising the virus disclosed herein. Other examples of the present disclosure relate to an immunogenic composition comprising the virus disclosed herein. Other examples of the present disclosure relate to a vaccine comprising the virus disclosed herein.

[0014] Other examples of the present disclosure relate to a composition containing an Enterovirus A virus produced by the method of any of the above examples. Other examples

of the present disclosure relate to an immunogenic composition containing an Enterovirus A virus produced by the method of any of the above examples. Other examples of the present disclosure relate to a vaccine containing an Enterovirus A virus produced by the method of any of the above examples.

BRIEF DESCRIPTION OF THE DRAWINGS

[0015] The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

[0016] FIG. 1 shows an experimental cell culture set-up in NANO. For the recirculation, a MasterFlex pump mounted with EasyLoad heads (not depicted) was used instead of the perfusion module shown in this picture.

[0017] FIG. 2A to 2C show pictures of the NANO carriers. Carriers were sampled out at different times and colored with Crystal Violet. **FIG. 2A** shows the empty carrier. **FIG. 2B** shows the carrier at Day 3. **FIG. 2C** shows the carrier at Day 6.

[0018] FIG. 3 shows monitoring results for the iCELLis NANO. The measured parameters were cell number, glucose and lactate concentrations, dissolved of oxygen (DO), pH, and temperature. Medium replacement was performed on Days 3 and 6.

[0019] FIG. 4 shows a comparison of cell densities in the iCELLis NANO and in the control Cell-Stacks. For the iCELLis NANO, cells were counted using the nucleus count method, whereas for the Cell-Stacks, cells were counted after trypsinization using the Trypan blue method.

[0020] FIG. 5 shows the experimental plan for the EV71 iCELLis NANO culture using a cellular density of 0.52 E6 cells/cm^2.

[0021] FIG. 6 shows the experimental plan for the control EV71 Cell-Stack culture using a cellular density of 0.52 E6 cells/cm^2.

[0022] FIG. 7 shows monitoring results for the EV71 iCELLis NANO culture using a cellular density of 0.52 E6 cells/cm^2. The measured parameters were cells number, glucose and lactate concentrations, dissolved of oxygen (DO), pH, and temperature. Medium replacement was performed on Day 3 and infection on Day 6.

CLAIMS

What is claimed is:

1. A method for producing an Enterovirus A virus, comprising:

(a) culturing an adherent cell in a fixed bed comprising a macrocarrier, wherein the cell is cultured in a first cell culture medium;

(b) inoculating the cell with the Enterovirus A virus under conditions in which the Enterovirus A virus infects the cell;

(c) culturing the infected cell in the fixed bed in a second cell culture medium under conditions in which the infected cell produces the Enterovirus A virus; and

(d) harvesting the Enterovirus A virus produced by the cell, wherein (i) the cell is inoculated with the Enterovirus A virus at a multiplicity of infection (MOI) of between 0.025 and 0.0009, optionally 0.001, and/or (ii) between 200,000 cells/cm^2 and 350,000 cells/cm^2 are inoculated in step (b).

2. The method of claim 1, wherein the cell is a Vero cell.

3. The method of any one of claims 1-2, wherein the Enterovirus A virus is selected from the group consisting of EV71, CA6, and CA16.

4. The method of claim 3, wherein the Enterovirus A virus comprises at least one non-human cell adaptation mutation.

5. The method of claim 4, wherein:

(a) the Enterovirus A virus is EV71, and wherein the at least one non-human cell adaptation mutation comprises a VP1 polypeptide mutation; or

(b) the Enterovirus A virus is CA6, and wherein the at least one non-human cell adaptation mutation comprises a mutation selected from the group consisting of a VP1 polypeptide mutation, a VP2 polypeptide mutation, a VP3 polypeptide mutation, and a 5' untranslated region (UTR) mutation; or

(c) the Enterovirus A virus is CA16, and wherein the at least one non-human cell adaptation mutation comprises a mutation selected from the group consisting of a 2A polypeptide mutation, a VP1 polypeptide mutation, a VP2 polypeptide mutation, and a 5' untranslated region (UTR) mutation.

6. The method of any one of claims 1-5, wherein between 4,000 cells/cm^2 and 16,000 cells/cm^2 are cultured in step (a).

7. The method of any one of claims 1-5, wherein between 200,000 cells/cm^2 and 350,000 cells/cm^2 are inoculated in step (b).

8. The method of any one of claims 1-7, wherein the first cell culture medium comprises serum.

9. The method of claim 8, wherein the first cell culture medium comprises less than 10% fetal bovine serum and the cell is not adapted to serum free medium.

10. The method of any one of claims 1-9, wherein the first cell culture medium and the second cell culture medium are different.

11. The method of claim 10, further comprising, between steps (a) and (b), removing the first cell culture medium and rinsing the cell with the second culture medium.

12. The method of any one of claims 1-11, wherein the second cell culture medium is a serum-free medium.

13. The method of any one of claims 1-12, wherein the cell is inoculated with the Enterovirus A virus at a multiplicity of infection (MOI) of between 0.025 and 0.0009.

14. The method of any one of claims 1-13, wherein the cell is cultured during steps (a), (b), and/or (c) at a volume/surface ratio of 0.3 mL/cm^2.

15. The method of any one of claims 1-14, wherein the lactate concentration in the first cell culture medium during steps (a) and/or (b) does not exceed 25 mM.

16. The method of any one of claims 1-15, wherein the lactate concentration in the second cell culture medium during step (c) does not exceed 25 mM.

17. The method of any one of claims 1-16, wherein the density of oxygen (DO) in the first cell culture medium during steps (a) and/or (b) is maintained above 50%.

18. The method of any one of claims 1-17, wherein density of oxygen (DO) in the second cell culture medium during step (c) is maintained above 50%.

19. The method of any one of claims 1-18, wherein the fixed bed has a bed height of 2 cm or 10 cm.

20. The method of any one of claims 1-19, wherein the macrocarrier is a microfiber matrix.

21. The method of any one of claims 1-20, wherein the macrocarrier has a porosity between 60% and 99%.

22. The method of claim 21, wherein the macrocarrier has a surface area accessible to the cell of between 150 cm^2/cm^3 and 1000 cm^2/cm^3.

23. The method of any one of claims 1-22, wherein at least 5.0 x 10^7 TCID50/mL of the Enterovirus A virus is harvested in step (d).

24. The method of any one of claims 1-23, further comprising inactivating the Enterovirus A with one or more of beta-propiolactone (BPL), formalin, or binary ethylenimine (BEI).

25. The method of any one of claims 1-24, wherein the Enterovirus A virus is an attenuated virus.