Enhancing enterovirus A71 vaccine production yield by microcarrier profusion bioreactor culture

Chia-Chyi Liu a,⇑, Suh-Chin Wu b, Shang-Rung Wu c, Hsiao-Yu Lin a, Meng-Shin Guo a, Alan Yung-Chih Hu a, Yen-Hung Chow a,d, Jen-Ron Chiang e, Dar-Bin Shieh c, Pele Chong a,d

a National Institute of Infectious Diseases and Vaccinology, National Health Research Institutes, Zhunan Town, Miaoli County, Taiwan
b Institute of Biotechnology, Department of Medical Science, National Tsing Hua University, Hsinchu, Taiwan
c Institute of Oral Medicine, College of Medicine, National Cheng Kung University, Tainan, Taiwan
d Graduate Institute of Immunology, China Medical University, Taichung, Taiwan
e Vaccine Center, Centers for Disease Control, Taipei, Taiwan

* Corresponding author at: Vaccine R&D Center, National Institute of Infectious Diseases and Vaccinology, Zhunan Town, Miaoli County 350, Taiwan.
E-mail address: 010113@nhri.org.tw (C.-C. Liu).

1. Introduction

Enterovirus A71 (EV-A71) is a non-enveloped single (+) strand RNA virus of the family Picornaviridae, genus Enterovirus. EV-A71 viral infections have caused several epidemics of hand, foot and mouth disease (HFMD) that were associated with severe neurological diseases in young children. It has become a serious public health problem in the Asia-Pacific region [1–6]. To prevent EV-A71 infections and periodic EV-A71 outbreaks, an effective vaccine against EV-A71 infection is urgently needed. Several EV-A71 vaccine candidates have been investigated including the live-attenuated virus, inactivated whole virus, recombinant viral protein, virus-like particle, recombinant plant, VP1 peptide and DNA vaccines [4]. Most of these EV-A71 vaccine candidates could induce various levels of virus neutralizing antibody responses in the mouse immunogenicity studies, but due to the success of polio vaccine and easy in regulatory issues, the chemical inactivated whole-virion EV-A71 vaccine candidates were first selected for clinical development [3,4,7].

The chemical inactivated EV-A71 vaccine candidates based on EV-A71 grown in the Vero cell culture using either roller bottle technology or cell factory technology have been tested in human clinical trials in Taiwan, mainland China and Singapore [8–14]. Serum-free media were used in the cell-culture to overcome the high serum-protein content that could complicate the downstream purification process and the risk of potential contamination with prions due to the bovine resource [15]. The whole-virion EV-A71 vaccine candidate either in empty virus particle (E-particle) or fully infectious virus particle (F-particle) could be either co-purified...
using the gel-filtration chromatography [8,9,13] or individually purified by sucrose gradient ultracentrifugation [14]. The ratio of the E-particle to the F-particle was 7:3 in the single batch culture [14]. To develop the large-scale manufacturing process for EV-A71 vaccine production, cells grown on the surface of a 5 g/L Cytodex 1 microcarrier and cultured in a serum-free bioreactor technology have been investigated and established [14,16,17]. Recently, a 200L-scale serum-free microcarrier bioreactor was successfully established to produce an EV-A71 vaccine candidate using a single-use bioreactor [18]. Their results show that the two types of EV-A71 viral particles were purified from the microcarrier bioreactor culture that could induce good immunogenicity against the EV-A71 virus.

Several parameters for viral vaccine production have been investigated that include cell lines, virus strains, culture media, bioreactor technologies and cultivation strategies [19]. In our previous studies, we used the following two-temperature culture method to increase the EV-A71 virus titer: the cell growth phase was at 37 °C and 32 °C for the infection phase [14,16,17]. The EV-A71 virus was released at higher titers in the supernatant than intra-cellular using microcarrier culture [16]. The glucose/glutamine feedings strategy could improve EV-A71 production yields during infection in batch culture [16], and this method has also been applied in a 200L-scale bioreactor [18]. Either roller bottle technology or microcarrier bioreactor technology for 200 L-scale cGMP-grade EV-A71 vaccine production requires over 2 months to amplify Vero cells. To increase vaccine yields, the conventional batch production system is modified with the fed-batch and perfusion methods for cell culture based manufacturing processes [19]. The perfusion method utilize feeding the fresh medium, while withdrawing the old culture medium to achieve higher cell concentrations and extended the bioreactor run times. The development of technically feasible large-scale manufacturing methods to enhance EV-A71 virus production yields could also reduce the production costs.

In this study, we present a medium replacement culture strategy as a good approach to improve EV-A71 virus production yields in the serum-free microcarrier bioreactor. Both the multi-harvested semi-batch culture (MHSBC) and perfusion culture technology in microcarrier culture were tested, and it was found that these technologies could enhance the production of the EV-A71 virus. This slow cell-death indicates that the semi-batch culture strategy and perfusion culture technology could be developed to enhance production yields in the scaled-up manufacturing processes. We also examined the immunogenicity of the EV-A71 antigens in mouse model. The results from the current studies would provide valuable information for developing large-scale serum-free microcarrier cell culture for manufacturing the chemical inactivated EV-A71 vaccine.

2. Materials and methods

2.1. Cells, culture medium, and virus

Vero cells (green monkey kidney cells) were kindly provided by the Taiwan Centers of Disease Control (Taiwan CDC), which were obtained from the original Vero cell line from the American Type Culture Collection (ATCC). Vero cells were grown in a VP-SFM medium (Gibco) and were passaged twice weekly in T-flasks. The EV-A71 E59 strain (genotype B4), the clinical isolate of the EV-A71 virus, was obtained from the Taiwan CDC [13]. EV-A71 viral stocks were harvested from the supernatants of infected Vero cells three days post infection (DPI). The titers of the virus stocks were determined by a TCID50 assay, and the stocks were stored at −80 °C.

2.2. EV-A71 virus cultivation using a bioreactor system

The EV-A71 virus was cultivated using serum-free VP-SFM medium in a BIOFLO 310 bioreactor (Eppendorf AG) based on the two-temperature microcarrier cell culture bioprocess as previously reported [14,16,17]. The bioreactor cultures (5 L of working volumes) were stirred at a rate of 60 rpm at pH 7, and the dissolved oxygen (DO) content was controlled at 50% by a gas mixer apparatus. The Vero cells used in the bioreactor were first harvested from the roller bottle and were detached using TrypLE select. Bioreactor culture medium containing 5 g of Cytodex 1 per liter was inoculated with 2 × 10⁶ cells per mL, and the cell density would reach to 2–2.5 × 10⁶ cells per mL after six days of cultivation. After a 70% culture medium replacement, the Vero cells were infected with the EV-A71 virus at a multiplicity of infection (MOI) = 10⁻⁵ [13,14]. EV-A71 virus stocks were harvested from the culture supernatants at 6–13 days post infection. The multi-harvested semi-batch culture method was defined as EV-A71 virus and was harvested by replacing 70% of the culture medium every two days and collected from the 6th, 8th, 10th and 12th DPI. A perfusion culture method was used for harvesting and collecting EV-A71 virus by replacing 50% of the medium every day from 7th to 13th DPI.

2.3. EV-A71 virus purification by continuous sucrose gradient ultracentrifugation

The upstream harvesting of the EV-A71 viral stocks was performed as previously described [14], the debris of the Vero cells was removed by a 0.65 μm filter (Sartorius), and viral stocks were concentrated using a 100 K TFF capsule ( Pall). The concentrated viral stocks were loaded to a 10–50% continuous sucrose gradient for ultracentrifugation at 32,000 rpm for 3 h using a zonal rotor in a Hitachi CP80 ultracentrifuge. These resultant fractions were subjected to SDS-PAGE and western analyses to detect purified EV-A71 viruses. The purified EV-A71 viral stocks were diaphragm using an Amicon 100 K tube (Millipore) by centrifuging at 3000g. The concentrated stock was re-suspended in PBS. The total protein concentration of the re-suspended viral stock was determined using a BCA protein assay (Pierce).

2.4. Determination of virus titers and specific titers

Virus titers were determined from the median end point of the tissue culture’s infectious dose (TCID50) according to the protocol described previously by Chang et al. [13]. The TCID50 values were calculated using the Reed-Muench methods. The specific titers were measured and defined as the total harvested virus yields after post-infection/total initially infected cell numbers produced under various experimental conditions.

2.5. SDS-PAGE analysis and western blotting

SDS-PAGE and western blot analyses of the purified EV-A71 virus fractions were performed in a NuPAGE 4–12% Bis-Tris Gel (Invitrogen) according to a previously described protocol [14]. An MAB979 monoclonal antibody (Millipore) was used to detect EV-A71 antigens. Novex® Sharp Pre-stained Protein Standards (ThermoFisher, LCS800) and PiNK Plus Prestained Protein Ladder (GeneDireX, PM005) protein markers were used in western blotting.

2.6. Characterization of viral particles by cryo-electron microscopy (cryo-EM)

The purified viral stocks were inactivated by 0.025% formaldehyde solution [14] and were adsorbed to grids coated with a holey
carbon film, blotted, and plunge-frozen in ethane using a Gatan CP3 Cryo Plunger. The frozen grids were observed and recorded with a JEM-2100F transmission electron microscope with an accelerating voltage of 200 kV. The micrographs were recorded on a 4 k × 4 k CCD camera (Gatan Ultrascan 4000 4 k × 4 k CCD Camera System Model 895). Viral particles were found to have diameters of approximately 30–35 nm.

2.7. Animal immunogenicity study

The animal protocol (NHRI-IACUC-101042-A) was reviewed and approved by the Institutional Animal Care and Use Committee, NHRI. Mouse immunogenicity studies were performed according to the protocols described previously [9,14]. Virus stocks were mixed with a 37% formaldehyde solution (Merck) at a of 4,000:1 volume ratio and stored for 3 days at 37 °C for virus inactivation. The aluminum phosphate (alum) was produced in NHRI cGMP facility [11]. Three groups of six female BALB/c mice (6–8 weeks old) were intraperitoneally (i.p.) immunized with 0.5 mL of PBS/alum (300 μg) as the control, the viral empty particle (alum-absorbed 0.25 μg antigen per dose), or the viral full particle (alum-absorbed 0.25 μg antigen per dose). Mice were boosted twice with the same dose at two week intervals after priming. The immunized mice were bled one week after the final boost, and the serum was collected.

2.8. Virus neutralizing assay

The sera collected from immunized mice were inactivated at 56 °C for 30 min. A virus neutralization assay was performed according to Liu et al. [14]. The TCID50 values were obtained by counting the cytopathic effects (CPE) in the infected Vero cells. The 50% neutralization inhibition dose that is the geometric reciprocal of the serum dilution yielding a 50% reduction in the viral titer was obtained using the Reed-Muench methods.

3. Results

3.1. EV-A71 virus culture in a bioreactor by perfusion culture methods

The preliminary MHSBC experiments were performed in 100-mL spinner culture system (Supplementary S1). To develop a feasible technical bioprocess for EV-A71 virus production, a 5-L working volume bioreactor was investigated. As the control, the single-batch method was performed in Vero cells grown on 5 g/L Cytodex 1 microcarriers reaching 2–2.5 × 10^8 cells/mL, 70% of the culture medium was replaced with a fresh VP-SFM medium and the cells were infected with the EV-A71 virus at an MOI = 10^{-5} in the bioreactor at 32 °C. Similar to the previous report [14], the viral titer was consistently found to be 0.7–1 × 10^6 TCID50/mL at either the 6th or 8th DPI harvested virus culture (Fig. 1A). In the MHSBC the medium was replaced at the 6th, 8th and 10th DPI, and the average viral titers from 3 different runs were found to be 13, 7, 4 and 0.7 × 10^6 TCID50/mL in the 6th, 8th, 10th and 12th DPI harvested virus culture media, respectively (Fig. 1A). From 3 different perfusion studies in which 50% of the culture medium was replaced every day after infection, the average viral titers found in the harvested virus culture were ranging from 1 to 2 × 10^6 TCID50/mL for the 7th to 13th DPI (Fig. 1A). The average harvested virus titers were calculated to be 2 × 10^6 TCID50/mL in the 4.5 L of a single batch culture, 5.8 × 10^6 TCID50/mL in a total 15 L of a semi-batch culture and 1.0 × 10^7 TCID50/mL in a total 18 L of perfusion culture. Both the MHSBC and the perfusion culture methods could significantly increase the virus production yields by 7–14-fold in comparison to those obtained from the single-batch culture method. The current perfusion technology certainly has more capability than the MHSBC culture method as measured by the specific titer (Fig. 1B). In addition, the current results indicate that the medium replacement strategy could provide savings in time and the cost of raw materials, such as microcarrier beads and a Vero cell bank seed.

To understand how the fresh culture media support virus production, the cell morphology and the CPE of the virus-infected Vero cells were examined after EV-A71 infection. The CPE was consistently observed starting at the 5th to 6th DPI and essentially all Vero cells were dissociated from microcarriers on the 8th DPI in the single-batch studies. In MHSBC studies, the CPE was also observed on the 6th DPI, but the Vero cells were slowly and gradually dissociated from microcarriers until the 12th DPI. In perfusion studies, CPE was also observed and the Vero cells were gradually dissociated from microcarriers during the 7th to 13th DPI. Taking the observation of CPE and the results of virus titers in the harvested virus culture together, it seems that EV-A71 virus replications maintain a stable rate as long as the cells still attach to

Fig. 1. EV-A71 E59 virus production in a microcarrier bioreactor culture. Vero cells cultured on 5 g/L microcarrier in 5 L working volumes and infected with the EV-A71 E59 strain at MOI = 10^{-5}. (■) In batch culture, there was no replacement. (○) In the MHSBC, 70% of the culture medium was replaced every two days on the 6th, 8th, 10th and 12th DPI. (▲) In perfusion culture, 50% of the culture medium was replaced every day on 7th to 13th DPI. (B) Specific titers of the EV-A71 E59 virus production. Specific titers were accumulated in different culture conditions.
the microcarrier beads. Additionally, the fresh culture medium certainly helps the cells continue to grow as the toxic materials derived from the dead cells are removed from the media. The EV-A71 virus is stable at 32 °C for a few days that provide enough times to operate MHSBC and perfusion culture methods.

3.2. Biochemical characterizations of virus collected from the MHSBC and perfusion culture media

We investigated whether the MHSBC and/or perfusion cell culture methods could produce EV-A71 virus particles that are similar to previous studies. The results described above indicate that consistent EV-A71 virus titers were produced from both the MHSBC and perfusion methods during the 6th to 13th DPI. Harvested virus culture from four time points (6th, 8th, 10th and 12th DPI) was individually concentrated and separated using a 10–50% continuous sucrose gradient ultracentrifugation into two types of EV-A71 virus particles as detected by the western blotting analysis (Fig. 2A). Using MAB979 VP2-specific monoclonal antibody, at all 4 time points, the EV-A71 E-particle characterized with a VP0 viral protein (36 kDa) was observed in Fractions #9 to #11, and the EV-A71 F-particle characterized with a VP2 protein (28 kDa) was revealed in Fractions #15 to #17. These results are identical to those observed from the single-batch bioreactor study reported previously [14]. A similar separation pattern of the virus particles was also detected by western blotting analysis of the 13th DPI harvested virus culture of the perfusion bioreactor run (Fig. 2B). The current results certainly confirm that the MHSBC and/or perfusion methods could produce two EV-A71 virus particles that are similar to the virus particles obtained from the single-batch culture or roller-bottle technology. We collected viral particles and evaluated the protein concentration from Fractions #9 to #11 (E-particle) and Fractions #15 to #17 (F-particle). The ratio of the two particles (E-particle/F-particle) was found to be 2.5 in the single-batch culture method, 1.7 in the MHSBC method and 1.4 in the perfusion method. In the roller-bottle technology, the ratio was found to be 2.3 in the single-batch production run. These results indicated both the MHSBC and perfusion methods could enhance more infectious virus yields from Vero cell.

3.3. Characterization of viral particles by cryo-EM

To have good preservation of structural features, the purified EV-A71 virus particles were individually inactivated with formalin (4000:1), suspended in vitreous ice and analyzed by cryo-EM (Fig. 3). The intact and spherical particles of the EV-A71 viruses are shown with a diameter of 30–35 nm in the cryo-EM micrographs, and their morphology was found to closely resemble those of the enteroviruses of the *Picornaviridae* family.

3.4. Immunogenicity study of formalin-inactivated EV-A71 virion derived from perfusion bioreactor

To determine the immunogenicity of the EV-A71 virion obtained from the perfusion technology using the 5-L bioreactor, the sucrose gradient ultracentrifugation purified virus stocks were treated with formaldehyde solution for virus inactivation as described by Liu et al. [14]. Mice were immunized with alum-absorbed formalin-inactivated EV-A71 virions, and the virus neutralization titers obtained a titer of 1280 using the E-particle

![Fig. 2. Purification of the EV-A71 virus from the bioreactor culture by sucrose gradient zonal ultracentrifugation. (A) Four multi-harvested semi-batch culture harvesting supernatants (6th, 8th, 10th and 12th DPI) were concentrated and separated into 25 fractions. Marker: Novex® Sharp Pre-stained Protein Standards (ThermoFisher). (B) The final perfusion culture harvesting supernatant (13th DPI) was concentrated and separated into 25 fractions. Marker: PiNK Plus Prestained Protein Ladder (GeneDireX). The EV-A71 antigens were detected by western blot using MAB979 VP2-specific monoclonal antibody. The VP0 protein (VP4 + VP2) was labeled at the expected molecular weight (●), and the VP2 protein was labeled at the expected molecular weight (□).](image)
immunization and 2560 using the F-particle immunization (Table 1). The PBS/alum control group had <8. The current results indicate that the inactivated EV-A71 virions prepared from the perfusion bioreactor culture technology can induce virus neutralizing titers that are comparable to those obtained from the batch bioreactor culture [9,14].

4. Discussion

The current EV-A71 vaccine candidates are produced from a virus grown in Vero cell using either the roller bottle or cell factory technologies as they are easy to implement and operate, and companies can establish the cGMP infrastructure and have a flexible facility for multiple applications [8]. However, these technologies are labor intensive and need a bigger facility to implement more roller bottles or cell factories in large-scale vaccine manufacturing. The microcarrier bioreactor technology can provide large-scale manufacturing in fixed or disposable bioreactors. The purpose of the current work is to develop a medium replacement culture strategy to increase EV-A71 virus production yields. This concept has been used in large-scale cell culture for viral vaccines, biopharmaceuticals and recombinant protein production [19–23,28].

The stirred tank bioreactors can be run in the following different modes: batch, fed-batch and perfusion culture [19,24]. The advantage of perfusion cultures is that cells are retained in the bioreactor, and the product is continuously harvested. Therefore, a smaller bioreactor can achieve higher productivities compared to those obtained in batch or fed-batch cultures. In addition, these culture methods are very easy to perform and do not need more expensive investments for the development of manufacturing processes. Perfusion culture has been successfully applied to improve rabies virus production [25,26] and flu virus production [23,27]. Cell culture based flu vaccine products are currently manufactured using perfusion culture system has recently been reviewed [23]. Microcarrier beads are designed for anchor-dependent cell lines in a stirred tank in which the culture parameters are easily monitored and controlled for cell growth. The microcarrier beads have a density of 1.04 g/mL and a mean diameter of 200 μm. These characteristics of the microcarrier are easy to keep in the bioreactor when the medium is replaced by gravity or a filter. In the current MHSBC, as the stirrer shuts down for 5–15 min, most microcarrier beads were settled in the bottom of the bioreactor. It was easy to harvest the virus supernatant and replace 70% of the fresh medium. It is worthwhile to note in the perfusion culture that a 70 μm mesh filter is set in the harvesting channel to enhance the virus production yield as the filter could prevent the cell-attached microcarriers from flowing through and being lost. However, there are also potential disadvantages regarding additional media costs, product stability and operational risks during the extended cultivation period.

In this work, we discovered that the infectious EV-A71 virus was consistently replicating when the fresh culture medium was replaced. Therefore, the medium replacement culture strategy works well for increasing EV-A71 virus production yields. After 6–7 DPI at the low MOI (10⁻⁵), the rate of CPE was slowed when a fresh medium replacement was performed. The MHSBC method could significantly increase the harvested virus production yields for EV-A71 in comparison with the single-batch culture method. We observed a 7–14 folds increase in the EV-A71 virus production yield in the perfusion culture technology. Two types of EV-A71 viral particles are produced in the cell culture production bioprocess. The F-particle ratio was increased in the medium replacement culture strategy. This method provides a feasible technical bioprocess for producing more F-particles.

The biochemical and biophysical analyses demonstrated that the MHSBC and perfusion culture methods produced a comparable EV-A71 particle pattern to the batch culture method (Fig. 2A and B). In cryo-EM observation, the inactivated EV-A71 virions prepared from the bioreactor culture technology were still maintaining a complete smooth particle structure (Fig. 3). EV-A71 structure proteins are assembled with viral RNAs to form F-particle, and E-particle is packed without viral RNAs [14]. In our previous studies, we detected a large amount of viral RNAs at F-particle fractions using real-time PCR [14]. Furthermore, a mouse immunogenicity study of the EV-A71 particles produced from a

Table 1

<table>
<thead>
<tr>
<th>Process</th>
<th>Sample</th>
<th>Total protein (μg)</th>
<th>The virus neutralization titer (GMT ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PBS/alum control</td>
<td>0</td>
<td>&lt;8</td>
</tr>
<tr>
<td>Perfusion bioreactor</td>
<td>E-particle</td>
<td>2.5</td>
<td>1280 ± 388</td>
</tr>
<tr>
<td></td>
<td>F-particle</td>
<td>2.5</td>
<td>2560 ± 675</td>
</tr>
<tr>
<td>Batch bioreactor^*</td>
<td>E-particle</td>
<td>2.5</td>
<td>906 ± 351</td>
</tr>
<tr>
<td></td>
<td>F-particle</td>
<td>2.5</td>
<td>2020 ± 799</td>
</tr>
</tbody>
</table>

^* This data is derived from our previous article [14]. To be consistent, the same starting materials (cell bank, virus seed, media and bioreactor) to culture and produce the EV-A71 virus particles were used.
perfusion bioreactor elicited the virus neutralizing titers that are comparable to those obtained from the batch bioreactor culture. These results showed that the medium replacement culture strategy did not change the basic biochemical properties, physical structures or immunogenicity of the EV-A71 particles.

5. Conclusion

The current results certainly confirm the success of the perfusion strategy and indicate that there are savings in time, the cost of the microcarriers and the usage of Vero cells. Therefore, the present study provides valuable information for the large-scale bioreactor manufacturing of the FI-EV-A71 vaccine.

Acknowledgements

This work was supported by the Ministry of Health and Welfare, the Ministry of Science and Technology (NSC 99A1-VCSP01-014), the Taiwan CDC and the National Health Research Institutes (02A1-IVPP12-014), Taiwan.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2017.02.042.

References

[20] Voisard D, Meuwly F, Ruffieux PA, Baer G, Kadouri A. Potential of cell retention and perfusion strategy and indicate that there are savings in time, the cost of the microcarriers and the usage of Vero cells. Therefore, the present study provides valuable information for the large-scale bioreactor manufacturing of the FI-EV-A71 vaccine.