Production of small ruminant morbillivirus, rift valley fever virus and lumpy skin disease virus in CelCradle™-500A bioreactors

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Abstract

Background: Animal vaccination is an important way to stop the spread of diseases causing immense damage to livestock and economic losses and the potential transmission to humans. Therefore effective method for vaccine production using simple and inexpensive bioprocessing solutions is very essential. Conventional culture systems currently in use, tend to be uneconomic in terms of labor and time involved. Besides, they offer a limited surface area for growth of cells. In this study, the CelCradle™-500A was evaluated as an alternative to replace conventional culture systems in use such as Cell factories for the production of viral vaccines against small ruminant morbillivirus (PPR), rift valley fever virus (RVF) and lumpy skin disease virus (LSD).

Results: Two types of cells Vero and primary Lamb Testis cells were used to produce these viruses. The study was done in 2 phases as a) optimization of cell growth and b) virus cultivation. Vero cells could be grown to significantly higher cell densities of 3.04 × 10⁹ using the CelCradle™-500A with a shorter doubling time as compared to 9.45 × 10⁸ cells in Cell factories. This represents a 19 fold increase in cell numbers as compared to seeding vs only 3.7 fold in Cell factories. LT cells achieved modestly higher cell densities of 6.7 × 10⁸ as compared to 6.3 × 10⁸ in Cell factories. The fold change in densities for these cells was 3 fold in the CelCradle™-500A vs 2.5 fold in Cell factories. The titers in the conventional system and the bioreactor were not significantly different. However, the Cell-specific virus yield for rift valley fever virus and lumpy skin disease virus are higher (25 virions/cell for rift valley fever virus, and 21.9 virions/cell for lumpy skin disease virus versus 19.9 virions/cell for rift valley fever virus and 10 virions/cell for lumpy skin disease virus).

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Background

Animal vaccination is an important way to minimize the spread of diseases that cause enormous damage to livestock, leading to a substantial economical impact. Viruses can be successfully contained by a well-organized vaccination, using sufficient coverage and effective vaccines, which prompted pharmaceutical industries to seek for flexible, cost efficient and operative production technology.

Animal vaccine strains are commonly cultured on adherent cells and less frequently on suspension cells for commercial purposes. Adherent cells such as Vero cells are frequently cultured on 2-D systems such as roller bottles and Cell Factories. but all of them include complicated operation and provide low population densities of cells. Furthermore, they are time consuming and involve heavy operations.

Microcarriers such as Cytodex have also been largely used for adherent cells, offering good mixing and oxygen transfer but often resulting in low cell densities due to accumulation of toxic metabolites and a high shear stress. To alleviate the shear stress problem, other reactors have been developed such as hollow fiber [1], packed-bed bioreactors [2] and Wave bioreactors [3]. In general, they have the advantages of good mixing, aeration and nutrient supply, but require an exterior oxygenation system and sophisticated operation skills.

A novel, single-use bioreactor -the CelCradle™-500A was evaluated in the current study for culture of adherent cells with the aim of cultivating viruses used in vaccine production for a number of veterinary diseases. This bioreactor for adherent cell culture has been re-evaluated in the current study for culture of adherent cells with the aim of cultivating viruses used in vaccine production for a number of veterinary diseases. The optimal cell density was achieved at different time points of the culture as shown in Fig. 1.

The kinetics of LT cell growth in the CelCradle™-500A system over a period of 10 days is represented in Fig. 2. 2.0 × 10⁸ cells/bottle was seeded at day 1 (D1), Peak cell densities were observed at Day 7 of culture with a total number of 6.7 × 10⁸ cells/ bottle and a drastic drop in cell numbers there after.

Conclusions: This work represents a novel study for primary lamb testis cell culture in CelCradle™-500A bioreactors. In addition, on account of the high cell densities obtained and the linear scalability the titers could be further optimized using other culture process such as perfusion.

Keywords: CelCradle™-500A, PPR virus, RVF virus, LSD virus, Vero cells, LT cells
A comparison is made between cell growth from cultures in conventional CFs and the CelCradle™-500A system (Table 1). In cell factories, the seeding density was 40,000 cells/cm² for both cell types. The cell density at harvest after 4 days/6 days post-seeding was 150,000 cells/cm² and 98,000 cells/cm² for Vero and LT cells respectively. This represents a 3.7-fold and 2.5-fold increase in cell numbers as compared to seeding. Vero cell growth increased by 19-fold in the CelCradle™ as compared to the seeding density whereas LT cells showed a modest increase of only 3–fold.

Vero cells had a doubling time (DT) of 24 h in Cell Factories versus 28 h in CelCradle system. For LT cells the DT was 76 h in Cell Factories and 86 h in CelCradle system.

Virus growth kinetics

PPR virus

As described previously, Vero cells grew optimally in medium containing 1% FBS. This condition was used for all subsequent experiments. Vero cells were inoculated when total cell numbers were $22 \times 10^8$ cells/bottle at D4 with PPR virus as described previously (Fig. 3a).

For the next 3 days, cell numbers did not increase (21.99, 22.01, and 22.03 cells/bottle). PPR virus titers however started to increase (from 4.4 to 5 log_{10} TCID50/ml) as represented in Fig. 3a below. At the time when cells start excreting virions, there was a drastic drop in cell numbers decreases $8 \times 10^8$ cells/bottle. In Fig. 3b we compared titers of the total extracellular and intracellular virus. There is a small difference between the two titers, 4 to 5 days post infection.

RVF virus

Figure 4a represents kinetics of RVF virus growth on Vero cells during 11 days of cultivation. Cells were inoculated with the virus when total cell numbers were $24 \times 10^8$ cells per bottle. As expected, cell numbers drop after inoculation with the virus as soon as 24 h post-inoculation and at day 5 show a drastic drop in cell numbers with a corresponding increase in viral titer. Figure 4b represents the titers of the total extracellular and intracellular virus. After 4 days, the extracellular virus had a titer of 7.6 log_{10} TCID50/ml and intracellular virus of $7.8 \log_{10} TCID50/ml$ after 5 days of inoculation.

LSD virus

Cells were inoculated at day 5 with the LSD virus at an MOI of 0.01 and when total cell numbers were $2.2 \times 10^8$ cells/bottle. At 4 dpi, LT cells reached their maximum cell concentration of $3.6 \times 10^8$ cells/bottle (Fig. 5a). The cells started to secrete LSD virions into the extracellular environment at this point. At D8 post infection, cells secrete the most number of virions as reflected by viral titers (6.9 log_{10} TCID50/ml) while cell densities dropped to $1.8 \times 10^8$cells/bottle.

At day 8, extracellular virus titer was $6.4 \log_{10} TCID50/ml$ whereas intracellular virus is $6.9 \log_{10} TCID50/ml$ (Fig. 5b).

Table 2 is a comparison of the titers of PPR, RVF and LSD viruses obtained by cultivation in 2 culture systems. In Cell Factories, the PPR titer was $6.3 \log_{10} TCID50/ml$ after 5 days of incubation, LSD was $6.5 \log_{10} TCID50/ml$ after 5 days of incubation and RVF was $7.8 \log_{10} TCID50/ml$ after 4 days of incubation. In CelCradle™ - 500A bioreactor, the PPR titer obtained was $6.4 \log_{10} TCID50/ml$ after 7 days of incubation, and LSD virus titers were $6.9 \log_{10} TCID50/ml$ after 8 days and $7.8 \log_{10} TCID50/ml$ for RVF after 5 days of incubation.

Discussion

Peste des Petits Ruminants, Rift Valley fever and Lumpy skin disease are among the most frequent and devastating diseases of livestock in Africa and Asia [12, 13, 15].
Those diseases can only be controlled by preventive measures through vaccination. As for RVF, vaccination is necessary not only to immunize animals but also to prevent animal to human transmission.

Most of the veterinary vaccines are produced in adherent cells; Vero cells are the preferred substrate for PPR and RVF viruses, and LT primary cells are the preferred substrate for LSD viruses [16–18]. Currently, these cells are cultured in open systems using Cell factories or roller bottles, which lead to a high risk of contamination. These systems have the added disadvantage of a large footprint and are labour-intensive as well. The conventional CF and roller bottles also involve lengthy handling operations, in addition CF provide poor oxygen transfer and present surface area limitations leading to low cell densities [9, 19]. Therefore, the development of an efficient adherent cell culture process is desirable.

Various types of bioreactors have been used for adherent cells like hollow fiber, packed-bed and disposable Wave bioreactors. They offer good oxygenation but they necessitate sophisticated skills to operate. In this study we evaluated the CelCradle™-500A system, for the production of LSD vaccine on LT primary cells and RVF and PPR vaccines on Vero cells. Our evaluation was based on (i) the comparison of the cell growth of the two type of cells in the CelCradle™-500A and Multitrays (ii) comparison of viral titers of the 3 respective viruses obtained in both systems.

To reduce serum percentage in growth medium for the culture of Vero cells, cells were cultured in DMEM with different percentages of FBS, (1, 3 and 5%). the most important cell attachment was achieved in 1% FBS medium with a total of 2.3 × 10^8 cells/bottle after 3 h of incubation.

Cell growth kinetics were evaluated for the 2 different cell types using conventional Cell Factories and the CelCradle™-500A. For Vero cells, a 19-fold increase in cell growth as compared to seeding density with a corresponding decrease in doubling time was observed. This is advantageous from the point of scale-up strategies and

|                      | Vero cells | LT cells |
|----------------------|------------|----------|
|                      | CelCradle™-500A | Cell Factory | CelCradle™-500A | Cell Factory |
| Inoculum (cell/ cm²) | 10,000     | 40,000   | 15,000     | 40,000     |
| Harvest (cell/ cm²) | 192,000    | 150,000  | 43,000     | 98,000     |
| Incubation time (days) | 5         | 4         | 8          | 6          |
| Fold-increase in cell numbers | × 19 | × 3.7 | × 3 | × 2.5 |
| Cell doubling time (h) | 28 h     | 24 h     | 86 h       | 76 h       |
| Medium volume (ml/cm²) | 32 ml     | 237 ml   | 32 ml      | 237 ml     |

Fig. 3 a Kinetics of Vero cell growth in 1% FBS-containing media and PPR virus titer. b Comparison of titers between extracellular and intracellular virus
to obtain higher virus titers as a result of high cell densities.

The CelCradle thus facilitates an enhancement in cell proliferation as compared to conventional culture systems such as the CF. In a similar study carried out in Japan with Vero cells by Hiroko et al. (2007), the author reported a total cell number of $28 \times 10^8$ cells/bottle of 7 days of incubation as compared to $30 \times 10^8$ cells/bottle in 5 days in our study [5]. Different types of microcarriers were used for Vero cells cultivation achieving a lower cell concentration: $1.35 \times 10^5$ cells/ml, $1.55 \times 10^5$ cells/ml, $2 \times 10^5$ cells/ml, $2.40 \times 10^5$ cells/ml, $4.7 \times 10^5$ cells / ml, $5 \times 10^5$ cells/ml, $1 \times 10^6$ cells/ml, $1.85 \times 10^6$ cells/ml, and $2.6 \times 10^6$ cells/ml [20–23].

Typical cell doubling time of Vero cells is 24 h [24], which is given by CF in our study. On CelCradle™-500A system we obtained a DT of 28 h, which is consistent with a relatively longer cell doubling time of 28–38 h in a study carried out by Yang et al. to examine Vero cells bead to bead transfer in spinner flasks with microcarriers. Therefore, it appears that, when the Vero cells were cultured on Cytodex, the doubling time of Vero cells was usually longer than 24 h [25]. In addition, Lai et al. (2019) reported a doubling time for Vero cells of...
LT cells were grown in the CelCradle™-500A to a total number of $6.7 \times 10^8$ cells/bottle after 8 days of culture; with CF’s, we obtained $6.23 \times 10^8$ cells/bottle after 6 days of culture. The CelCradle therefore yields a few higher cell numbers than the CF’s. To date, very few studies have reported efficient cultivation of primary cells using the CelCradle™-500A system. Chen et al. in (2016) differentiated rat pancreatic duct-derived stem cells (PDSCs) and successfully obtained after 10-fold increase in cell density at the end of the culture period of 7 days [10]. In 2007 S. Frauenschuh et al. cultivated primary mesenchymal stem cells on Cytodex microcarriers given a cell concentration of 10,000/14 ml [26].

The CF has an added disadvantage in that after 6 days of culture, cell numbers dropped drastically and showed a steep decrease in viability. This is due to pH regulation difficulty, low aeration and metabolites limitation. Such problems were not encountered in CelCradle™-500A cultures.

In this work, we studied kinetics of three viruses on two different production systems. The obtained viral titers were similar in both systems for PPR and RVF viruses. As for LSD virus, the titer was higher in CelCradle™-500A as compared to CF by $4.78 \times 10^8$ infectious units of virus/ml. The run time was one to 3 days longer in CelCradle™-500A than in CF, it was also reported to be 6 days longer by Lewis Ho et al. in 2004, where it was shown that the cultivation of HEK 293 cell line using CelCradle system took 12 days using CelCradle system versus 6.5 days using Cell Factories system [9].

The virus characteristics and impact on the cell substrate was variable in the 3 viruses cultivated in the CelCradle. PPR and RVF inoculation block cell growth immediately after infection whereas after LSD virus infection, cells continue their growth for 2 days before a drop in viability. This can be explained by the fact that LSDV is an intracellular virus with slow replication as compared with PPR and RVF. For the 3 viruses, we observed that intracellular titer is higher than the extracellular one. This could be explained by the fact that the virus in the supernatant is diluted in the medium.

Conventional culture systems for adherent cells tend to reduce cell growth because of their space limitation and design. The CelCradle™-500A system offers many advantages at different levels. The 3D environment increases surface area for culture, a uniform distribution of cells and maximum aeration and nutrition by virtue of the “Tide motion” principle. This provides low shear stress, high aeration with no O2 limitation, and a foam-free culture environment. Thus, optimum cell density and consequently, high viral titers are possible. Moreover, macrocarriers—the matrices for cell growth allows the adherence of Vero cells in low serum medium, which reduces the cost of the production.

Owing to the simple design, this system is extremely easy to handle and operate and can be used for small-scale vaccine production and for preparation of seed trains. These benefits enable it to become a simple and economical system for high-density cell culture and virus production. It has been successfully utilized to grow primary cells, and to produce PPR, RVF and LSD vaccines.

Viral titers were comparable in both systems. Since all our trials were done without medium replenishment, these titers could be optimized by the usage of a perfusion system, and this being a continuous system could enhance volume of virus harvested and possibly the titer as well.

### Conclusion

Taking into consideration that the CelCradle™-500A is a laboratory scale bioreactor which yields titers equivalent to Cell factories that are conventional industrial scale systems, this represents a promising preliminary study for potential use of a novel bioreactor. It important to mention that cultures in the the Tide motion bioreactors are linearly scalable and production of the 3 viruses reported here can be readily scaled up in TideXcell 2-5000 L bioreactors.

### Methods

**Cells and viruses**

Vero cells were purchased from ATCC (no. CCL-81) and initially cultivated in 1, 3 or 5% of foetal bovine serum (FBS)-containing DMEM medium for the purpose of selecting the optimal conditions for further cell cultivation and virus production experiments. Primary lamb testis cells LT, were obtained by castration of a healthy 3 month old male and obtaining cells from the testis. Cells were prepared and propagated in Dulbecco’s modified

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| Viruses | Incubation period (Days) | Virus titer ($\log_{10}$TCID50/ml) | Cell-specific virus yield (virions/cell) |
|---------|--------------------------|------------------------------------|-----------------------------------------|
|         | CelCradle™-500A | Cell Factory | CelCradle™-500A | Cell Factory | CelCradle™-500A | Cell Factory |
| PPR     | 7           | 5               | 6.4          | 6.3          | 1.29        | 6.33         |
| RVF     | 5           | 4               | 7.8          | 7.8          | 25          | 19.9         |
| LSD     | 8           | 5               | 6.9          | 6.5          | 21.9        | 10           |

Table 2: Comparison of viruses titers and incubation time using Cell Factories and CelCradle systems
Eagle’s medium supplemented with 10% FBS. Three attenuated vaccine strains were used in this study: PPRV Nigeria 75 strain [27], RVF Clone 13 T virus [28] and LSD virus Neethling strain [29].

**Bioreactor system**

The CelCradle™ - 500A is a single-use bioreactor capable of yielding high-density cell cultures for production of vaccines, recombinant proteins and monoclonal antibodies. The bioreactor used in this study consists of two compartments; an upper chamber made of polyethylene terephthalate containing 5.5 g of macrocarriers which provide the matrices for cell adherence and growth and a lower compressible chamber (LCC) of low-density polyethylene containing the medium. The cap is equipped with a 0.22 μm PTFE filter.

The CelCradle was mounted on a stage and the parameters for cultivation were set using a control unit. The upward and downward movement of the media provides a “Tide motion”. Cells on the macrocarriers thus receive an alternating cycle of aeration and nutrition.

**Cell culture**

Macrocarriers were equilibrated in 400 ml of media. Following this, 100 ml of the respective cells was added to individual CelCradle™-500A bioreactors.

Cell seeding and attachment period was for 3 h. After this period of incubation, 2 macrocarriers were sampled using sterile forceps, fixed with 2 ml of 95% ethanol, stained with 2 ml of Trypan blue, and were observed microscopically to visualise cell attachment. After determining that the cell attachment was more than 90%, the Tide motion parameters were changed to cell cultivation. The Tide motion parameters of cell seeding and cell cultivation were as follows:

| Tide motion | Rising rate | Top holding time | Down rate | Bottom holding time |
|-------------|-------------|------------------|-----------|---------------------|
| Cell attachment | 2 mm/sec | 20 s | 2 mm/sec | 0 s |
| Cell cultivation | 1.5 mm/sec | 20 s | 1.5 mm/sec | 0 s |

Cell density on carriers was also evaluated by a crystal violet dye (CVD) nucleus staining method with 2 macrocarriers taken from the bottle. The carriers were incubated at 37°C with 1 ml of CVD and vortexed every 15 min. A hemocytometer was then used to count the nuclei as a readout for the number of cells.

The cell doubling time was calculated with the following formula:

\[
\text{Cell doubling time (DT)} = \ln 2 / \mu, \quad \text{Where } \mu = \ln X_n / \ln X_{n-1}/t_n - t_{n-1}.
\]

Experiments were executed in 2 phases: Phase 1 and Phase 2 for optimizing cell-growth and virus culture respectively. Initially, the cell growth kinetics was investigated for Vero cells. Optimum FBS concentrations for Vero cell culture was determined using 1, 3 and 5% serum in the medium. The optimal medium was then used for subsequent virus propagation experiments. For phase 1, seeding cell concentration for Vero cells was \(11.36 \times 10^3\) cells/cm² for each of the 3 types of medium.

In the phase 2 of virus production, cell seeding was \(22.72 \times 10^3\) cells/cm².

For LT cells, the feasibility of growing them on the carriers was determined before subsequent experiments. Cell concentration used was \(1.7 \times 10^3\) cells/cm². This was essential to determine if indeed primary cells could be cultivated on the carriers. For the virus production experiments, cell seeding of LT cells was \(2.77 \times 10^3\) cm².

In parallel as a head-to-head comparison, Cell Factories (CF, Nunc 10 chamber) with a total surface area of 6320 cm², were seeded with \(3.99 \times 10^4\) cells/cm² LT cells or Vero cells (with 1500 ml of DMEM supplemented with 5% FBS) and incubated at 37°C with 5% CO₂.

**Virus production**

Culture medium in each of the 3 bottles CelCradles was replaced by 300 ml of DMEM containing 1%FBS. Cells were inoculated with viruses at an MOI of 0.01 on day 4 for PPR and RVF and on day 5 for LSD virus, already adapted to the respective cells. The titers of the seeded viruses were 6.2 (log₁₀TCID50/ml) for PPR, 7.5 (log₁₀TCID50/ml) for RVF and 6.3 (log₁₀TCID50/ml) for LSD.

After a virus adsorption period of 3 h, 200 ml more of medium was added to each of the CelCradles.

The Tide motion parameters were changed to the following:

| Rising rate | Top holding time | Down rate | Bottom holding time |
|-------------|------------------|-----------|---------------------|
| 1.0 mm/sec | 20 min | 1.0 mm/sec | 0 s |

**Virus titration**

Two samplings of the macrocarriers were carried out daily until D10 post infection in order to determine the virus growth kinetics. Two milliliters of the supernatant culture medium containing secreted virus, extracellular virus, was obtained at each sampling and stored at 4°C. Two
macroribbons were also sampled for intracellular virus and stored at – 20 °C until titration. The intracellular virus was obtained by lysing the cells by a freeze/thaw cycle.

For virus titration assays, Vero/LT cells were seeded at a density of 110, 000 cells/well and (100 µl) of serially diluted virus was added to each well. After an incubation period of 96 h at 37 °C, the virus titer was determined by IPMA assay as described by Andy et al, 2020 [30].

Abbreviations
PPR: Small ruminant morbillivirus; RVF: Rift valley fever virus; LSD: Lumpy skin disease; LT: Lamb tests; DT: Doubling time; LCC: Lower compressible chamber; CVD: Crystal violet dye; CF: Cell Factory

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Authors’ contributions
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Ethics approval and consent to participate
This research didn’t involve animal trials.

Consent for publication
this manuscript didn’t include details, images, or videos relating to an individual person.

Competing interests
The authors declare that they have no competing interests.

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