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Propagation of Brazilian Zika virus strains in static and suspension cultures using Vero and BHK cells

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ABSTRACT

The recent spread of Zika virus (ZIKV) in the Americas and the Pacific has reached alarming levels in more than 60 countries. However, relatively little is known about the disease on a virological and epidemiological level and its consequences for humans. Accordingly, a large demand for *in vitro* derived Brazilian ZIKV material to support *in vitro* and *in vivo* studies has arisen. However, a prompt supply of ZIKV and ZIKV antigens cannot be guaranteed as the production of this virus typically using Vero or C6/36 cell lines remains challenging.

Here we present a production platform based on BHK-21 suspension (BHK-21$_{SUS}$) cells to propagate Brazilian ZIKV at larger quantities in perfusion bioreactors. Scouting experiments performed in tissue culture flasks using adherent BHK-21 and Vero cells have demonstrated similar permissivity and virus yields for four different Brazilian ZIKV isolates. The cell-specific yield of infectious virus particles varied between respective virus strains (1–48 PFU/cell), and the ZIKV isolate from the Brazilian state Pernambuco (ZIKV^{PE}) showed to be a best performing isolate for both cell lines. However, infection studies of BHK-21$_{SUS}$ cells with ZIKV^{PE} in shake flasks resulted in poor virus replication, with a maximum titer of 8.9×10^3 PFU/mL. Additional RT-qPCR measurements of intracellular and extracellular viral RNA levels revealed high viral copy numbers within the cell, but poor virus release. Subsequent cultivation in a perfusion bioreactor using an alternating tangential flow filtration system (ATF) under controlled process conditions enabled cell concentrations of about 1.2×10^7 cells/mL, and virus titers of 3.9×10^7 PFU/mL. However, while the total number of infectious virus particles was increased, the cell-specific yield (3.3 PFU/cell) remained lower than determined in adherent cell lines. Nevertheless, the established perfusion process allows to provide large amounts of ZIKV material for research and is a first step towards process development for manufacturing inactivated or live-attenuated ZIKV vaccines.

1. Introduction

Zika virus (ZIKV) is an arthropod-borne, enveloped virus belonging to the *Flaviviridae* family and the flavivirus genus. This family encompasses more than 75 single-stranded RNA viruses like Yellow fever virus, Japanese Encephalitis virus, West Nile virus and Dengue virus. These viruses are primarily transmitted by *Aedes* mosquitoes. In addition, sexual transmission seems likely based on clinical and serologic evidence [1]. Sporadic infections of humans with ZIKV have been reported in Africa from 1952 onwards, and circulation of the virus has resulted in natural population immunity over time. Therefore, observed progression of disease was rather mild or, due to low frequency and missing documentation, misinterpreted [2]. In contrast, the ZIKV outbreak in the Americas spread rapidly over the continent and has been accompanied by associated cases of microcephaly and Guillain–Barré syndrome [3,4]. Limited information regarding ZIKV disease, the association to fetal malfunctions and the lack of preventive or therapeutic tools prompted the World Health Organization (WHO) to declare it a global health emergency [5]. At present, the transmission of ZIKV and the mechanisms underlying ZIKV-related microcephaly and other neurodevelopment defects remain poorly understood and higher quantities of *in vitro* virus material should be produced as fast as possible to establish quantitative assays and to enable more comprehensive virological and immunological studies [6].

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Current strategies for propagation of African and Brazilian ZIKV in animal cells mainly rely on Vero (kidney fibroblasts from African green monkey) cells and mosquito C6/36 (*Ae. albopictus* larvae) cells. The African ZIKV strain MR 766 has shown reasonable titers in both cells, whereas Brazilian ZIKV isolates from the current outbreak do not seem to propagate in similar quantities applying standard infection protocols (personal communication, A. Tanuri). Vero cells have an extraordinary safety profile and are widely used as an approved substrate for the production of human viral vaccines at commercial scale [7], whereas lineages of the mosquito C6/36 cell line have been used only in fundamental research involving arboviruses [8]. However, both anchorage-dependent cell lines are difficult regarding large scale vaccine manufacturing as trypsinization is required for scale-up and passaging. Accordingly, the identification of a suspension cell line capable of producing reasonable virus titers would be a significant contribution towards establishment of a platform for ZIKV vaccine manufacturing.

In this study, we investigated Brazilian ZIKV replication in Vero and BHK-21 (kidney fibroblasts from Syrian baby hamsters) cells to develop a lab-scale process for the production of virus material in quantities required for the establishment of assays and virological studies. Therefore, we have adapted adherent BHK-21 cells to growth in a chemically defined (CD) medium and suspension. This enabled the scale-up into perfused and controlled stirred tank bioreactors to achieve high cell concentrations and reasonable virus yields even in the case low cell-specific yields cannot be avoided.

2. Materials and methods

2.1. Cell lines and cultivation conditions

Vero E6 cells (ATCC® CRL-1586™) were maintained in tissue culture flasks with DMEM (high glucose, Gibco, USA, #11965092) and 5% fetal bovine serum (Sigma, USA, #F7524). Porcine kidney stable epithelial (PS) cells (thankfully provided by M. Niedrig, Robert-Koch Institute, Germany) were used for the plaque assay and were grown in GMEM (Gibco, USA, #22100093) containing 10% fetal bovine serum, 2% FMV Peptone (LaB-M, UK, #MC033), 2 mM L-glutamine and 2 mM pyruvate (hereafter referred to as Z-Medium). BHK-21 C13 cells (kindly provided by IDT Biologika GmbH, Germany) were initially cultivated in Z-Medium, but then step-wise adapted to suspension growth in the CD medium TCX6D or in equal volumes of TCX6D with TC-LECC medium (Xell AG, Germany), both supplemented with 8 mM L-glutamine. The basal growth medium TCX6D/TC-LECC for the perfusion run was additionally supplemented with 100 IU/mL Pen-Strep (Gibco, USA, #15140148). The adapted BHK-21$_{SUS}$ cells were cultivated in spin-tubes with vented caps (TPP Sigma-Aldrich, USA, #Z761028) at 40 degree angle or in baffled shake flasks using an orbital shaker with 25 mm shaking diameter at 200 rpm (Celltron, Infors AG, Switzerland).

2.2. Zika virus propagation

Brazilian ZIKV strains have been isolated from whole blood specimens of PCR-positive adult patients from different regions in Brazil during the acute phase of the recent ZIKV outbreak. ZIKV^{ES} and ZIKV$^{ES.F}$ were isolated in Espírito Santo state, ZIKV^{PE} in Pernambuco state and ZIKV^{PB} in Paraiba state. All of them were initially propagated in C6/36 insect cells or in Vero E6 cells using tissue culture flasks and a virus stock was generated (virus material kindly provided by A. Tanuri, UFRJ; A. B. Filippis, Instituto Oswaldo Cruz, Fiocruz; and E. Caride, Bio-Manguinhos, Fiocruz). For further infection experiments, adherent cells grew to 90% confluency, when the supernatant was discarded and cells were infected at a multiplicity of infection (MOI) between 0.01 and 0.001 in reduced medium volume (10%). After 1 h virus adsorption, fresh medium was added and cells incubated for up to six days. Routinely, BHK-21$_{SUS}$ cells in shake flasks were directly infected during the mid/late exponential growth phase with different ZIKV isolates at an MOI of 0.001 without medium reduction or medium exchange. Virus samples were collected, centrifuged at 2000*g* for 3 min to remove cell debris, and supernatant was stored at −80 °C until use.

2.3. Zika virus quantification

Virus titers of the supernatant were determined by plaque assay. Therefore, diluted virus samples and PS cells (4×10^5 cells/well) were added simultaneously to 24-well plates and incubated for 4 h at 37 °C. The cell/virus mixture was overlaid with 1.6% (*w/v*) carboxyl-methyl-cellulose in Z-Medium and incubated for three days. Cells were then fixed with 3.7% (*v/v*) formalin in phosphate-buffered saline (PBS) for 15 min and stained with napthalin black (1 g naphthol blue black, 13.6 g sodium acetate, 60 mL glacial acetic acid, add to 1 L ddH_2O) for 30 min (adapted from [9]). Plaques were counted and titers expressed as plaque-forming units per volume (PFU/mL) in accordance to Spearman and Kärber [10]. Intra- and extracellular viral RNA (vRNA) levels were determined by quantitative reverse transcription PCR (RT-qPCR). In brief, intact suspension cells were centrifuged at 200*g* for 5 min. The supernatant was used for extracellular vRNA quantification. The cell pellet was washed once with PBS to remove remaining extracellular virus and re-suspended in fresh PBS. Intracellular vRNA was released into the supernatant by three freeze/thaw-cycles of the re-suspended cells. After an additional centrifugation step at 2000*g* for 3 min, cell debris was removed and the supernatant containing intracellular vRNA was further processed. Intra- and extracellular vRNA molecules were extracted with QIAmp MiniElute Virus Spin (Qiagen, Netherlands, #57704) following the manufacturer's protocol. A set of primers and a probe specific for the E protein of ZIKV (previously described in [11]) was used with One-Step TaqMan RT-PCR Master mix reagents (Applied Biosystems, USA, #4309169). Quantification was performed with a 7500 Real-Time PCR System (Applied Biosystems) following the manufacturer's recommendations.

2.4. Perfusion cultivation in 3 L single-use bioreactors

Cultivation was performed in a perfusion bioreactor system using a disposable Mobius® 3 L stirred tank vessel (Merck Millipore, USA, #CR0003L200), an ez-control unit (Applikon, Netherlands), and an alternating tangential flow filtration system (Xcell ATF 2, Repligen, USA). pH and dissolved oxygen (DO) probes as well as the perfusion dip-tube with pump housing and PES hollow fiber filter (0.2 µm pore size, 1 mm ID, 0.13 m^2 surface area, Repligen, USA, #F2:RF02PES) were autoclaved and connected under sterile conditions to the bioreactor vessel. The cultivation was performed in 1.2 L working volume (wv) at a stirrer speed of 120 rpm (pre-configured marine impeller) and a DO set-point of 80% air saturation maintained by pulsed sparging with pure oxygen and a maximum flow rate of 0.1 L/min. The ATF module was started with pump parameters, set points and process ranges as given by the supplier before inoculation to ensure homogenous cultivation conditions. BHK-21$_{SUS}$ were inoculated at a starting concentration of 7×10^5 cells/mL. At day 5, 0.7 reactor volumes (RV) were exchanged with basal growth medium to avoid depletion of media components. Afterwards, continuous perfusion of medium was started with supplemented perfusion medium (1:1 TCX6D/TC-LECC; 16 mM L-glutamine and 1× MEM non-essential amino acids

solution, Gibco, USA, #11140050) at a daily RV exchange rate (RV/d) of 0.15 and increased to 0.42 at the end of cultivation to maintain glucose concentrations above 5 mM. When a cell concentration of about 1.2×10^7 cells/mL was obtained, perfusion was stopped and 30% of the cell-free medium was removed through the hollow fiber filter. Subsequently, concentrated cells were infected with ZIKV^{PE} at an MOI of 0.001 with periodic stirring (3 min on, 15 min off) to facilitate virus infection of cells. After 1 h virus adsorption, the stirrer was set back to 120 rpm and the bioreactor was filled up with fresh basal growth medium to the initial working volume. Perfusion was switched on after 3 h (0.15 RV/d) to keep glucose concentrations above 5 mM, while pH was maintained at 7.1 by adding 0.5 M NaOH until the end of the process to maintain optimal cell growth conditions and to reduce pH-induced virus inactivation.

2.5. Sampling and analytics

Cell concentration and cell viability for spin-tube and shake flask experiments were determined by Muse Cell Analyzer with Count & Viability Assay Kit (both Merck Millipore, USA, #MCH100102). Same data for bioreactor cultivations were determined by manual trypan blue staining and counting using an optical microscope and a hemocytometer. Glucose concentrations were measured with measurements strips (Accu-Chek Active, Roche, Switzerland, #GG03149574).

3. Results

3.1. Brazilian ZIKV isolates differ strongly in virus yields

To characterize the replication of different Brazilian ZIKV strains, virus titers and cell-specific yields were determined in adherent BHK-21 and Vero cells (Table 1). For both cell lines, the maximum virus titers and the cell-specific yields were in the same order of magnitude. In contrast, the different virus isolates varied significantly in their titers. For example, in adherent BHK-21 cells, the maximum titer varied in the range of 4.1×10^5 PFU/mL to 1.3×10^7 PFU/mL, and the cell-specific yields from 1 to 41 PFU/cell. A similar variation was observed for the adherent Vero cells. Clearly, in this direct comparison, the best yields for BHK-21 cells were obtained with ZIKV^{PE}, which makes this virus-host cell system a candidate for virus production.

3.2. Restricted virus propagation in BHK-21$_{SUS}$ cells

To study ZIKV replication in suspension cells, BHK-21 cells were adapted to a CD medium and to surface-independent growth (suspension). Subsequent infection studies in small scale spin-tubes following a factorial experimental design enabled the identification of optimal infection parameters. Highest virus titers of ZIKV^{ES} isolates were obtained for infections at mid/late exponential cell growth phase (data not shown), whereas changes in MOI only had a minor impact on maximum yields (Fig. S1). Accordingly, an MOI of 0.001 was chosen for all subsequent experiments. When BHK-21$_{SUS}$ cells were infected after three days, cells grew exponentially with a population doubling time (t_D) of 25 h which strongly decreased to a t_D of 125 h after infection (Fig. 1, left). The infection experiment resulted in a maximum concentration of 7.1×10^6 cells/mL and a maximum virus titers not exceeding 9×10^3 PFU/mL (80 h post infection).

To further investigate the low ZIKV propagation in BHK-21$_{SUS}$ cells, intra- and extracellular vRNA levels were measured by RT-qPCR to identify potential bottlenecks in virus release (Fig. 1, right). The linear correlation of extracellular cycle threshold (c_T) values to infectious titers in the supernatant determined by plaque assay

Table 1
Maximum virus titer and cell-specific yields of Brazilian ZIKV strains in adherent BHK-21 and Vero cells.

ZIKV strain	BHK-21 (MOI 0.01)		Vero (MOI 0.001)	
	Max. virus titer (PFU/mL)	Cell-specific yield[a] (PFU/cell)	Max. virus titer ($\times 10^5$ PFU/mL)	Cell-specific yield[a] (PFU/cell)
ZIKV^{PE}	1.3×10^7	40.8	1.6×10^7	48.1
ZIKV$^{ES.F}$	3.1×10^6	9.9	2.8×10^6	8.9
ZIKV^{ES}	4.1×10^5	0.9	1.2×10^6	4.0
ZIKV^{PB}	2.9×10^6	9.3	n.d.	n.d.

[a] Based on maximum cell concentration. n.d. = not done due to limited virus availability. Coefficient of variation (%CV) of ≤15%. Virus material derived from Vero or C6/36 cell. Previous experiments showed that the maximum virus yields were independent from the chosen MOI (as indicated in Fig. S1). We therefore assume the present infection study as comparable in its results.

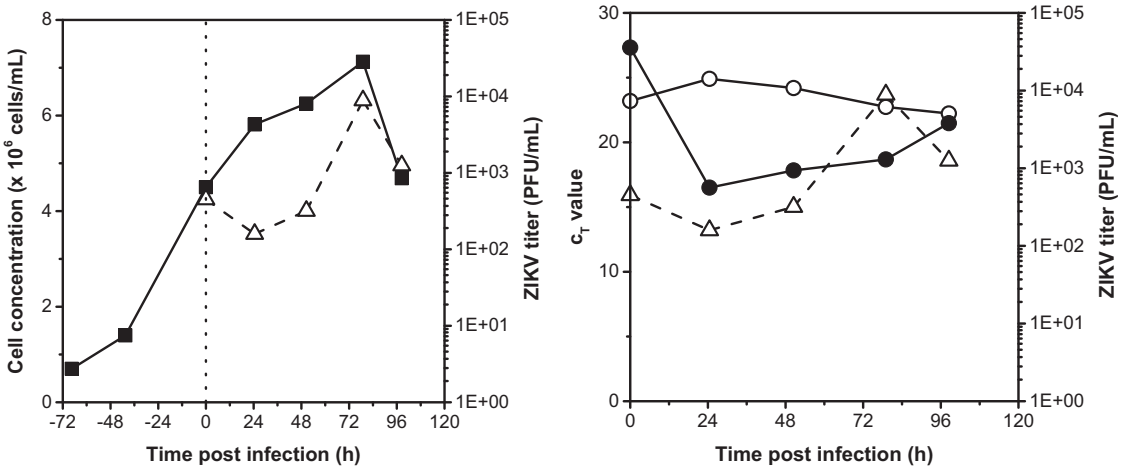

Fig. 1. Growth and infection of BHK-21$_{SUS}$ cells with ZIKV^{PE} at an MOI of 0.001. (Left) Shake flask cultivation of BHK-21$_{SUS}$ and infection during late exponential growth phase at an MOI of 0.001. Cell growth (■), ZIKV^{PE} titer (△). Vertical dotted line indicates time of infection. (Right) RT-qPCR analysis of intra- (●) and extracellular (○) vRNA levels in comparison to the ZIKV^{PE} titer (△).

(Fig. S2) enabled the interpretation of intracellular vRNA levels and the relative retention of virus material. Within one day, c_T values of the intracellular vRNA levels dropped significantly (indication of successful virus entry and vRNA synthesis in BHK-21$_{SUS}$ cells) and started to increase over the virus propagation phase again (decrease of intracellular vRNA levels). When the maximum virus titer of 9×10^3 PFU/mL in the supernatant was measured (80 h post infection), the intracellular RNA level was about 2000-fold higher than the extracellular level (its c_T value was lower), indicating non-optimal virus release.

3.3. Higher cell density cultivations

With the low cell-specific virus yields obtained for BHK-21$_{SUS}$ cells in shake flasks, very large volumes would be required to produce larger quantities of ZIKV material. To overcome this limitation, perfusion cultivation using an ATF2 system was established to achieve higher cell concentrations in a next step. Cells grew within eight days to a maximum concentration of 1.2×10^7 cells/mL and reached an average t_D of 84 h (Fig. 2). To avoid limitations in glucose concentration, 0.7 reactor volumes were withdrawn five days after inoculation and replaced by fresh basal medium. Afterwards, a continuous medium exchange was initiated and low perfusion rates (8–21 mL/h) were sufficient to maintain the glucose concentrations above 5 mM. Prior to infection at day 8, BHK-21$_{SUS}$ cells were concentrated in the bioreactor and subsequently infected with ZIKV^{PE} at an MOI of 0.001 as described above. During the following two days, the cell concentration remained stable at 10.5×10^7 cells/mL before concentrations dropped significantly due to virus-induced cell lysis. A maximum virus titer of 3.9×10^7 PFU/mL was achieved four days post infection. Based on the maximum cell concentration at time of infection, this corresponded to a cell-specific virus yield of 3.3 PFU/cell. A total volume of 3.5 L medium was used for the perfusion over the period of 9.5 days. The cut-off of the filter membrane (200 nm) used was considerably larger than a ZIKV particle (50 nm), but only 23% of the virus concentration passed the filter at the maximum. When the highest titer was achieved at day four post infection, titers of 7×10^6 PFU/mL were observed in the permeate relating to 20% of the virus concentration in the bioreactor. The low permeate flow rate resulted in an infectious virus loss of 4%. Towards the end of the cultivation, the RV/d was increased to 0.42, and viral titers in the permeate dropped to 4.5×10^4 PFU/mL (data not shown).

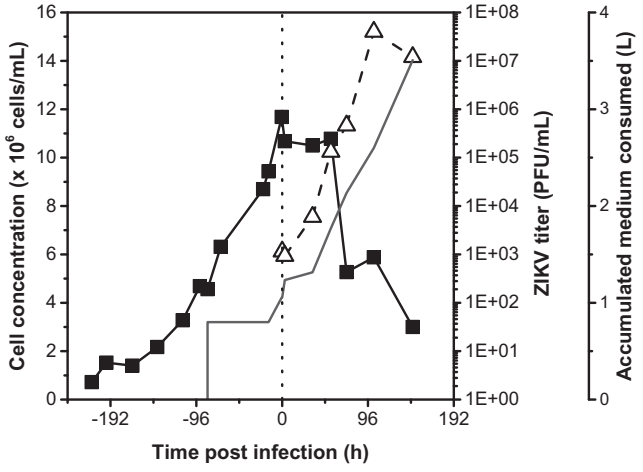

Fig. 2. Perfusion bioreactor cultivation for production of ZIKV^{PE} in BHK-21$_{SUS}$ at higher cell densities. Time course of BHK-21$_{SUS}$ cells (■), ZIKV^{PE} titers (△), and volume of medium consumed for perfusion (solid line). Dotted line indicates time of infection.

4. Discussion

4.1. ZIKV and their yields in animal cell culture

Current ZIKV propagation protocols typically rely on adherent Vero and C6/36 insect cells. In this study, adherent BHK-21 and Vero cells showed similar yields for the four Brazilian virus isolates tested. However, the maximum virus yields varied by two orders of magnitude, indicating a strong influence driven by their different origins and histories (patient, region, passage history). Notably, our results suggest that isolates have to be chosen carefully for the production of larger virus amounts for assay development and clinical studies. Besides their immunogenic properties, host cell system and cell-specific yields need to be evaluated critically regarding the design of production processes. Further adaptation of virus isolates, identification of optimal infection conditions, and choice of media may allow to further improve yields of current ZIKV strains. Deeper understanding may derive from genome sequencing of different isolates to reveal the linkage of mutations and their impact on their replication in cell culture.

4.2. ZIKV propagation in BHK-21$_{SUS}$ cells

BHK-21$_{SUS}$ cells showed low doubling times of 25 h achieving concentrations more than 7×10^6 cells/mL in shake flasks and simple batch mode. In addition, they are easy to handle in orbitally shaken or mechanically stirred cultivation vessels, which makes them an ideal candidate for large scale production. Infection studies with the best performing ZIKV^{PE} isolate, however, showed significantly reduced cell-specific yields compared to adherent BHK-21 cells. This may be ascribed to both, differences in media composition (lack of serum) and in cell morphology (anchorage-independent cell maintenance). However, as the chosen CD medium does not support adherent cell growth, this cannot be further explored. To overcome the low virus titers in BHK-21$_{SUS}$ cells cultivated in CD medium, first experiments focused on the improvement of virus adsorption in suspension culture. Hence infections were performed by centrifugal spinoculation [12], high MOI (>5) or in decreased cultivation volumes at time of infection. However, none of these approaches resulted in significant improvements (Fig. S3). To follow-up on a possible assumption of non-optimal virus maturation and virus release in BHK-21$_{SUS}$ cells, RT-qPCR was used to evaluate vRNA levels. At the time point maximum titers were achieved (80 h post infection), the intracellular number of vRNA molecules was 2000-fold higher than in the supernatant, which may be related to poor virus release and low extracellular viral titers. This finding is in agreement with transmission electron microscopic observations, where ZIKV was identified to remain in cellular vesicles inside adherent Vero cells [13]. In addition, it is not clear, whether all cells were infected and contributed to virus yields. Further studies using flow cytometry and staining of viral antigens should help to answer these critical questions regarding process design and optimization [14].

4.3. ZIKV^{PE} titers in the perfusion bioreactor

One strategy to overcome process limitations due to low cell-specific virus yields is process intensification by increased cell concentrations. Thus, BHK-21$_{SUS}$ cells were maintained in a bioreactor coupled to an ATF2 cell retention system. Controlled process parameters and continuous medium exchange enabled cell concentrations of up to 1.2×10^7 cells/mL in a single-use Mobius system. Although the metabolic activity of BHK-21$_{SUS}$ cells was higher in stirred bioreactor systems than in shake flasks (data not shown),

the average doubling time (84 h) was lower compared to a 3 L Applikon glass bioreactor and non-controlled shake flasks (50 h and 25 h, respectively, Fig. S4). However, it has to be taken into account that perfusion experiments with the Mobius system had to be carried out under BSL-3 conditions and that limited possibilities of monitoring metabolite concentrations may have resulted in suboptimal cell growth.

Nevertheless, within four days virus titers increased significantly to 3.9×10^7 PFU/mL in the perfusion bioreactor. Although the cell-specific yield (3.3 PFU/cell) was about 10-times lower in comparison to adherent BHK-21 cells, the controlled cultivation clearly improved total virus production and one cultivation in the ATF system could replace more than 90 tissue culture flasks with 175 cm^2 and 40 mL wv each (3.6 L in total). Despite the slow-lytic nature of flavivirus release, a sharp drop in cell concentration was observed two days post infection. This may have enabled the release of viral particles so that further infections were initiated. Furthermore, maintaining optimal cultivation conditions might have positively influenced the cell status for virus replication and stable pH values at 7.1 might have supported pH-depended virus maturation and diminished virus inactivation [15] so that high virus titers were achieved. However, the drop in cell-specific yields in comparison to adherent cells remains unclear. Although a filter membrane with 200 nm cut-off was chosen, only a small fraction of ZIKV particles (50 nm) migrated through the filter. One day post infection, 20% of the virus concentration in the bioreactor was found in the permeate flow and strongly decreased to less than 1% to the end of the cultivation. Similar results were previously obtained for influenza virus [16]. Details about unspecific virus adsorption onto the filter membrane or membrane clogging remain to be investigated. Future studies with different filter cut-offs, virus retention or virus harvest strategies with membrane stacks could be equally considered to design production processes.

4.4. Use of BHK-21 cells for human vaccine production

Continuous adherent BHK-21 C13 cells are well-known for their robust cell growth, their adaptability towards suspension growth and high cell densities. The cultivation of BHK-21 in suspension culture and their susceptibility for various virus families enables large scale manufacturing of veterinary vaccines at affordable prices, like rabies vaccines [17] and foot and mouth disease vaccines [18]. Additionally, BHK-21 cells are used in the industrial production of recombinant biopharmaceuticals, such as human FVIII and FVIIa, and is therefore considered as a well-characterized cell substrate by the WHO [19]. Despite the long history of success, the use of BHK-21 cells for human vaccine production is highly controversial as intact cells have been classified tumorigenic almost 40 years ago [20]. However, with the significant advances made in downstream processing regarding the depletions of DNA and host cell proteins, continuous cell lines, such as BHK-21 cells, should not necessarily be excluded for vaccine production purpose anymore [19]. Clinical studies of an inactivated BHK-derived rabies vaccine in humans showed great efficacy and a satisfactory safety profile [21,22]. Before the acceptance and establishment of BHK-21 as cell substrate for human vaccine production, it will require further costly purity, safety and consistency studies [19], but over the long run advantages such as high permissivity for many virus families, high productivity, robustness, and fast growth to high concentrations in suspension culture may turn these studies commercially attractive. For the meanwhile, virus material produced in adherent or suspension BHK-21 cells could be used to deliver enough ZIKV material for assay development as well as virological and epidemiological studies.

5. Conclusions

Current cell-culture based ZIKV production relies on Vero and C6/36 cell lines. However, the surface-dependent growth of both cell lines and the low cell-specific yields of Brazilian ZIKV isolates limit the establishment of large scale production processes to provide virus material for assay development and scientific studies. We have infected adherent BHK-21 and Vero cells with different Brazilian ZIKV isolates and observed that cell-specific virus yields were independent from the host cell. However, besides this intrinsic consistency of each isolate, virus titers strongly varied between different ZIKV isolates. This led to the identification of a high producer virus isolate, namely $ZIKV^{PE}$. Infection studies of $BHK\text{-}21_{SUS}$ cells with $ZIKV^{PE}$ indicated possible limitations in virus release observed by accumulation of intracellular vRNA molecules. Higher virus yields were obtained by the use of a perfusion bioreactor that enabled a significant increase in cell concentrations. The characterization of intracellular virus replication and virus spreading using flow cytometry as well as the optimization of infection conditions, media, and cultivation conditions will help to identify bottlenecks in virus production and support the design of improved production processes.

Conflicts of interest

The authors declare no conflict of interest.

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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.2017.03.018.

References

[1] Foy BD, Kobylinski KC, Chilson Foy JL, Blitvich BJ, Travassos da Rosa A, Haddow AD, et al. Probable non-vector-borne transmission of Zika virus, Colorado, USA. Emerg Infectious Diseases 2011;17:880–2.
[2] Kindhauser MK, Allen T, Frank V, Santhana RS, Dye C. Zika: the origin and spread of a mosquito-borne virus. Bull World Health Organ E-pub. 9.02.2016 ed2016.
[3] Cao-Lormeau V-M, Blake A, Mons S, Lastère S, Roche C, Vanhomwegen J, et al. Guillain-Barré Syndrome outbreak associated with Zika virus infection in French Polynesia: a case-control study. The Lancet 2016;387:1531–9.
[4] Mlakar J, Korva M, Tul N, Popović M, Poljšak-Prijatelj M, Mraz J, et al. Zika virus associated with microcephaly. N Engl J Med 2016;374:951–8.
[5] WHO. WHO statement on the first meeting of the International Health Regulations (2005). Emergency Committee on Zika virus and observed increase in neurological disorders and neonatal malformations. <http://www.who.int/mediacentre/news/statements/2016/1st-emergency-committee-zika/en/#>; 2016 [25.08.16].
[6] WHO. One year into the Zika outbreak: how an obscure disease became a global health emergency. <http://www.who.int/emergencies/zika-virus/articles/one-year-outbreak/en/>; 2016 [25.08.16].

[7] Gallo-Ramirez LE, Nikolay A, Genzel Y, Reichl U. Bioreactor concepts for cell culture-based viral vaccine production. Expert Rev Vaccines 2015;14:1181–95.
[8] Walker T, Jeffries CL, Mansfield KL, Johnson N. Mosquito cell lines: history, isolation, availability and application to assess the threat of arboviral transmission in the United Kingdom. Parasites Vectors 2014;7:382.
[9] Bae H-G, Nitsche A, Teichmann A, Biel SS, Niedrig M. Detection of yellow fever virus: a comparison of quantitative real-time PCR and plaque assay. J Virol Methods 2003;110:185–91.
[10] Hierholzer JC, Killington RA. 2 - Virus isolation and quantitation A2 - Mahy, Brian WJ. In: Kangro HO, editor. Virology methods manual. London: Academic Press; 1996. p. 25–46.
[11] Lanciotti RS, Kosoy OL, Laven JJ, Velez JO, Lambert AJ, Johnson AJ, et al. Genetic and serologic properties of Zika virus associated with an epidemic, Yap State, Micronesia, 2007. Emerg Infect Dis 2008;14:1232–9.
[12] O'Doherty U, Swiggard WJ, Malim MH. Human immunodeficiency virus type 1 spinoculation enhances infection through virus binding. J Virol 2000;74:10074–80.
[13] Barreto-Vieira DF, Barth OM, da Silva MAN, Santos CC, Santos ADS, Batista FJ, et al. Ultrastructure of Zika virus particles in cell cultures. Mem Inst Oswaldo Cruz 2016;111:532–4.
[14] Frensing T, Kupke SY, Bachmann M, Fritzsche S, Gallo-Ramirez LE, Reichl U. Influenza virus intracellular replication dynamics, release kinetics, and particle morphology during propagation in MDCK cells. Appl Microbiol Biotechnol 2016;100:7181–92.
[15] Janis AM, Mirja H, Axel S, Stephanie J, Detlef M, Thomas M, et al. Inactivation and Environmental Stability of Zika Virus. Emerg Infect Dis J 2016;22.
[16] Genzel Y, Vogel T, Buck J, Behrendt I, Ramirez DV, Schiedner G, et al. High cell density cultivations by alternating tangential flow (ATF) perfusion for influenza A virus production using suspension cells. Vaccine 2014;32:2770–81.
[17] Pay TW, Boge A, Menard FJ, Radlett PJ. Production of rabies vaccine by an industrial scale BHK 21 suspension cell culture process. Dev Biol Stand 1985;60:171–4.
[18] Radlett PJ, Pay TW, Garland AJ. The use of BHK suspension cells for the commercial production of foot and mouth disease vaccines over a twenty year period. Dev Biol Stand 1985;60:163–70.
[19] WHO. Annex 3 - Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks. (WHO Technical Report Series). <http://www.who.int/biologicals/vaccines/TRS_978_Annex_3.pdf>; 2013. 978 [13.09.16].
[20] Reid LM, Jones CL, Holland J. Virus carrier state suppresses tumorigenicity of tumor cells in athymic (Nude) mice. J Gen Virol 1979;42:609–14.
[21] Lalosevic D, Lalosevic V, Lazarevic-Ivanc L, Knezevic I. BHK-21 cell culture rabies vaccine: immunogenicity of a candidate vaccine for humans. Dev Biol (Basel) 2008;131:421–9.
[22] Lalosevic D, Stankov S, Lazarevic-Ivanc L, Lalosevic V, Knezevic I. Immunogenicity of BHK-rabies vaccine in human volunteers. Med Pregl 1998;51(Suppl 1):17–9.