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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

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