Vaccine xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Increasing Vero viable cell densities for yellow fever virus production in stirred-tank bioreactors using serum-free medium

Diogo A. Mattos[a,1], Marlon V. Silva[a,1], Luciane P. Gaspar[a,*], Leda R. Castilho[b]

[a] Bio-Manguinhos—FIOCRUZ, Technological Development Vice-directory, Rio de Janeiro, RJ, Brazil
[b] Federal University of Rio de Janeiro (UFRJ), COPPE, Chemical Engineering Program, Rio de Janeiro, RJ, Brazil

ARTICLE INFO

Article history:
Available online xxx

Keywords:
Yellow fever virus production
Vero cells
Serum-free medium
Bioreactors

ABSTRACT

In this work, changes in Vero cell cultivation methods have been employed in order to improve cell growth conditions to obtain higher viable cell densities and to increase viral titers. The propagation of the 17DD yellow fever virus (YFV) in Vero cells grown on Cytodex I microcarriers was evaluated in 3-L bioreactor vessels. Prior to the current changes, Vero cells were repeatedly displaying insufficient microcarrier colonization. A modified cultivation process with four changes has resulted in higher cell densities and higher virus titers than previously observed for 17DD YFV.

1. Introduction

Yellow fever is an acute disease transmitted by mosquitoes of the genera *Aedes* or *Haemagogus* and caused by the yellow fever virus (YFV), which belongs to the genus *Flavivirus* of the family *Flaviviridae* [1,2]. Endemic in most of South America and sub-Saharan Africa, 200,000 cases are estimated to occur annually, with a 20–50% fatality rate. There are no specific therapies and vaccination is the most effective way to prevent and control the disease [2,3].

The current available vaccine against yellow fever consists of live attenuated virus and provides a good and long-lasting protection with a good safety and efficacy profile [4,5]. However, since the 1990s, some cases of severe adverse effects have been reported, mainly after large-scale vaccination campaigns [6–8].

As previously described for influenza vaccine, the current yellow fever vaccine production process is slow, labor-intensive and hard to scale-up, therefore requiring the maintenance of large strategic stockpiles for the case of epidemics [9].

The development of a production platform based on animal cell cultivations in bioreactors would be useful for the production of new vaccine candidates, either attenuated or inactivated ones [10]. The new vaccine produced by this technology would be an alternative product in accordance with the current trends for biotechnological products for human use.

2. Materials and methods

2.1. Cell line, culture medium and cell cultivation protocols

Vero cell line CCL 81 was obtained from the European Collection of Animal Cell Cultures (ECACC). Culture medium was VP-SFM (Gibco, Invitrogen Corp.) supplemented with 4 mM Glutamax (Gibco, Invitrogen Corp.). A working Cell Bank had been produced after adaptation of cells to serum-free medium, as cells were originally growing in serum-containing DMEM. Bioreactor runs were carried out in a BioFlo 110 system (New Brunswick Scientific, model M1273-0054), fitted with a 3-L vessel (1–2.2 L working volume). Temperature was kept at 37 °C, pH at 7.2, dissolved oxygen at 50% or 20% air saturation and agitation at 80 rpm. Cytodex-1 concentration was 3 or 1.5 g/L, and the working volume was 2.2 L. To protect cells from sparging and agitation, 0.1% (m/v) Pluronic F68 (Sigma-Aldrich Co.) was added to the medium in all experiments.

Two different cell cultivation protocols have been evaluated. The original protocol used 3 g/L Cytodex I, one impeller, dissolved oxygen setpoint at 50% air saturation, medium added all at once (after cell seeding and viral infection) and final harvest performed at 96 h post-infection (hpi). The modified protocol employed 1.5 g/L Cytodex I, two marine type impellers (installed on the same axis), dissolved oxygen set for 20% air saturation, stepwise medium addition and final harvest at 72 hpi. Impellers were marine type and provided upwards axial flow.

Vero cells were inoculated at 0.2×10^6 cells/mL in both protocols. In the modified one, cells were seeded in a reduced volume of 1 L and medium was added 24 and 48 h after inoculation, when maximal working volume of 2.2 L was reached.

* Corresponding author. Tel.: +55 21 3882 9317; fax: +5521 2260 4727.
E-mail address: lgaspar@bio.fiocruz.br (L.P. Gaspar).
[1] Both authors have equally contributed to this publication.

http://dx.doi.org/10.1016/j.vaccine.2015.04.050
0264-410X/

2.2. Infection conditions

Infection was performed as described before [9]. Prior to infection, which happened 72 h after cell inoculation, agitation was turned off to allow microcarrier sedimentation and removal of 80% of medium. New complete medium was added to the volume of 1 L, and infection occurred at reduced volume. Multiplicity of Infection was 0.02 and the appropriate volume of the virus seed stock was added to the bioreactor using a syringe (<5 mL). In the original protocol, after 4 h of viral infection, cultivation volume was taken to its maximal by complete medium addition up to the maximal bioreactor working capacity. In the modified protocol, 0.6 L of complete medium was added in the following 24 and 48 h, similarly to the cell growth phase.

2.3. Analytical methods

Samples were taken daily to assess cell count and, after infection, virus production as well. To measure the total cell concentration, nuclei staining with crystal violet was carried out as previously described [11]. After centrifugation of 1 mL samples at 300 × *g* for 3 min, the supernatant was removed and the same volume of a 0.1% (m:v) crystal violet solution was added. Following vortexing, samples were maintained at 37 °C for 1 h. Appropriate dilutions were prepared and stained cell nuclei were counted in a Neubauer chamber, in triplicates. Viable cell density was calculated by subtraction of the dead cell density determined by trypan blue exclusion. After infection, samples of supernatants were assayed for virus titer by plaque titration on Vero cell monolayers, as described previously [12], and for protein E content by an Enzyme-Linked Immunosorbent Assay (ELISA) assay, developed in house, which measures the virus envelope protein concentration. For the ELISA assays, 96-well plates were coated with anti-YFV antibodies in Phosphate-buffered saline (PBS) buffer containing 3% (v:v) fetal calf serum, 5% (m:v) milk lecithin, 0,05% (m:v) Bovine Serum Albumin (BSA) and 0,05% (m:v) Tween. After incubation of samples, peroxidase-conjugated anti-YFV antibodies were used as detection antibodies and revelation was performed with 3,3',5,5'-Tetramethylbenzidine (TMB). The same ELISA assay protocol for 17DD YFV has also been used in a previous work for establishment of downstream methodology [13].

3. Results and discussion

3.1. New cultivation protocol for higher cell densities

Our previous studies described YFV yields of 10^8 pfu/mL in cultures of Vero cells using serum-free medium in bioreactors with working volumes of up to 1 L [10]. In the current work, a modified process is described for Vero cells cultivation in 2.2-L working volume, in which culture medium is gradually added to the vessels, until total volumetric capacity of vessels is achieved. This reduces foam formation and microcarrier aggregation in the bioreactors. Microcarriers concentration employed was also reduced to reproduce the cells/cm^2 ratio used in pre-inoculum cultures.

Five independent YFV production batches under the conditions of the modified process showed consistency in terms of cell growth kinetics and volumetric viral yield, with satisfactory cell density at the time of viral infection (>10^6 cells/mL) and viral titers higher than previously obtained in the 1-L scale process. These results suggest that the new production protocol is adequate and scalable, with no deleterious effects on volumetric viral yield, and indicates the feasibility of further scaling-up of YFV production.

The main objective of the cell growth phase (pre-infection) is to obtain the highest possible viable cell density (VCD), as viable cells

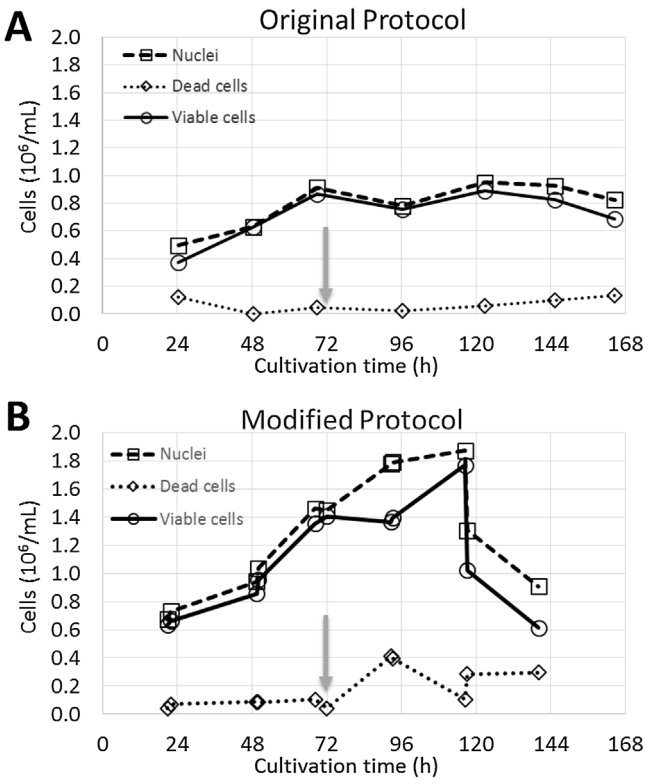

Fig. 1. Vero cell cultivation in serum-free medium in bioreactors (2.2 L working volume). (A) Vero cell growth kinetics according to the original protocol. (B) Vero cell growth kinetics according to the modified protocol. Gray arrows indicate time of infection.

are the crucial substrate for viral replication in the virus production phase (post-infection). The higher the VCD at the moment of infection, the more likely to obtain high virus yields after the infection phase [14]. To achieve high VCDs, some research groups have chosen to use customized media, which deliver very good results, as medium performance is thoroughly tested and optimized for each cell line and cultivation mode. Rourou et al. have obtained over 5 × 10^6 cells/mL with a proprietary medium for Vero cells cultivation and by recirculating medium [15]. The VCDs found in the present study are in agreement with the ones described by Genzel et al. [16], using commercial serum-free medium.

In this work, it was observed that better performance in cell growth was achieved by introduction of some new conditions: reduced volume in cell inoculation phase, which improved cell attachment to microcarrier surface; less foam and aggregates accumulation due to the use of two impellers; decreased stress through macrosparging, as the DO setpoint had been reduced, decreasing the demand for gas sparging. Fig. 1 exemplifies this finding, where a typical run of each protocol is depicted. Even with less microcarriers, higher cell densities were achieved.

Table 1 contains data from runs previously performed under the original protocol which was developed by Souza et al. [10]. The

Table 1
Summary of five 17DD production batches performed according to the original protocol. VCD_{max}: Maximal viable cell density during cultivation. Viral titers: viral titration results expressed in $\log_{10}$(pfu/mL).

Batch	VCD_{max} (10^6 cells/mL)	Viral titers ($\log_{10}$(pfu/mL))	Harvest time
A	0.85	7.0	192 h (120 hpi)
B	0.98	7.3	168 h (96 hpi)
C	0.78	7.3	168 h (96 hpi)
D	0.85	7.8	168 h (96 hpi)
E	0.90	8.2	168 h (96 hpi)

Please cite this article in press as: Mattos DA, et al. Increasing Vero viable cell densities for yellow fever virus production in stirred-tank bioreactors using serum-free medium. Vaccine (2015), http://dx.doi.org/10.1016/j.vaccine.2015.04.050