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Process intensification for an enhanced replication of a newly adapted RM-65 sheep pox virus strain in Vero cells grown in stirred bioreactor

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ABSTRACT

Sheep pox virus initially adapted to replicate in primary lamb kidney cells was adapted to Vero cells by serial passages in monolayer cultures. After nine passages the virus was able to correctly replicate in Vero cells, virus titer achieved was $10^{5.875}$ $TCID_{50}$ (median tissue culture infective dose) ml^{-1}.

To optimize the production process, the effects of MOI (multiplicity of infection), TOI (time of infection) and the culture medium were investigated. Cell infection at a MOI of 0.005 concurrently with cell seeding showed the best results in terms of specific virus productivity. The effect of MEM enrichment with several components was investigated using the experimental design approach. 67 experiments were performed in 6-well plates to select the best combination. The highest titer was achieved when MEM was supplemented with 5 mM glucose, 5 mM fructose and 25 mM sucrose. Spinner culture confirms these data; virus titer was $10^{7.375}$ $TCID_{50}$ ml^{-1}.

In addition Vero cells were cultivated in a 7-l bioreactor in batch mode on 3 g l^{-1} Cytodex1, and infected at cell seeding at a MOI of 0.005. Maximal virus titer was $10^{7.275}$ $TCID_{50}$ ml^{-1}. This corresponds to 44-fold factor enhancement compared to spinner cultures conducted in MEM + 2% FCS.

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1. Introduction

Sheep occupy an important place in the livestock industry and contribute significantly to the world economy. Their populations are threatened by a number of health hazards among them sheep pox [1]. This disease is an OIE notifiable [2] and transboundary disease of sheep and goats [3]. The etiological agent, sheep pox virus belongs to the genus *Capripoxvirus* in the subfamily *Chordopoxvirinae* of the Poxviridae family.

Sheep pox disease is economically important in the enzootic regions of Africa, the Middle East, Central Asia and the Indian subcontinent [4–7]. The disease is characterized by pyrexia, generalized skin lesions, internal pox lesions, and lymphoadenopathy. Following an incubation period of 4–8 days, there is an increase in body temperature, heart rate and respiration, followed by the formation of macules in the skin [3,4,8]. These ultimately develop into pox lesions, which can affect over 50% of the skin surface. These lesions contain high viral loads [9] and together with mouth lesions serve as the main source of virus for transmission to uninfected animals. Outbreaks of sheep pox, with very high morbidity and mortality rates, have been reported from different parts of the world regularly [4,10]. The mortality rate in young animals can exceed 50%. In naive animals, morbidity and mortality can even approach to 100% [4,10].

Due to the rapid progression of the diseases among livestock animals, treatment is generally not possible or practical, and the emphasis is placed on prevention by vaccination [4,5]. The control of the disease is vital to improve small ruminant productivity. Various kinds of vaccines are available but the live attenuated vaccine is the best choice. Live attenuated vaccines provide long-lasting immunity and, hence, are considered the best choice for use in vaccination [4,7,11]. The use of inactivated vaccine suffers from several drawbacks such as the use of high doses, and short duration of immunity [1,4,7].

Various cell types such as lamb testes, kidney and thyroid, calf kidney and fetal muscle, fetal sheep kidney, lung and skin tissues and continuous cell lines (BHK-21, Vero) have been the most commonly employed systems for virus attenuation and replication [4,11–13].

A live attenuated sheep pox vaccine was locally produced in primary lamb kidney cells for sheep immunization in Tunisia. Nevertheless, this vaccine provoked outbreaks of Border Disease and abortion in ewes after vaccine administration. Deep investigations demonstrated the contamination of this vaccine with *Pestiviruses*

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[14]. Therefore the production of this vaccine was interrupted; and the development of a safer sheep pox vaccine was initiated. For this purpose, Vero cells were selected as a cell support for sheep pox virus replication. This cell line is considered as the most widely accepted continuous cell lines by regulatory authorities for the manufacture of viral vaccines such as polio [15], rabies [16,17] and Japanese encephalitis [18] vaccines. These developments have been encouraged by the broad sensitivity of Vero cells to many viruses [19] and by the ability of this cell line to replicate a wide variety of virus strains to high titers [20–23].

In this work, we will first describe the adaptation of the primary lamb kidney cells adapted RM-65 sheep pox virus strain to replicate in Vero cell line; then we will describe our approach for the intensification of production of the newly adapted sheep pox virus strain in Vero cells grown on Cytodex1 microcarriers under stirred conditions.

2. Materials and methods

2.1. Cell line

Vero cells at passage 131, provided by the National Laboratory for Control of Biologicals (Tunis, Tunisia) and originally obtained from ATCC (CCL-81), were used in this study.

2.2. Virus strain

RM-65 virus strain was kindly provided by Razi Institute (Teheran, Iran). The strain was adapted to grow in primary lamb kidney cells for 30 passages.

2.3. Culture media and reagents

Media and fetal calf serum (FCS) were supplied by Invitrogen (Glasgow, UK). Reagents were purchased from Sigma–Aldrich (St Louis, USA). Wheat gluten peptone (Hypep 4601) was provided by Sheffield-Bioscience (Norwich, NY, USA).

2.4. Microcarrier preparation

Cytodex 1 from GE Health Care (Uppsala, Sweden) was used throughout this study. They were prepared and sterilized according to the manufacturer instructions.

2.5. Monolayer cell cultures

Cells were maintained in 75 cm^2 T-flasks in MEM + 10% FCS at 37 °C and 5% CO_2. Once a week, cells were trypsinized and subcultivated at 8×10^4 cells cm^{-2}.

2.6. Roller bottle cultures

Cells were cultivated in MEM + 10% FCS at 37 °C, seeded at a cell density of 10^5 cells cm^{-2} and infected at the required MOI. Cells were grown in 850 cm^2 roller bottles containing 100 ml of culture medium. Four days post inoculation cells were washed twice with MEM + 2% FCS and temperature was decreased to 34 °C. Agitation was kept at 3 rpm.

2.7. Spinner cultures

Cells were first grown in MEM + 10% FCS and 3 g l^{-1} Cytodex1. Once cell density reached the maximum, cells were washed twice with MEM + 2% FCS and infected with the sheep pox virus strain at a MOI ranging from 0.001 to 0.005. Cell growth and virus production phases were performed at 37 °C and 34 °C, respectively. Cultures were carried out in 250 ml spinner containing 200 ml of culture medium and inoculated at 2×10^5 cells ml^{-1}. Agitation rate was equal to 30 rpm, pH was maintained at 7.2 (cell growth phase) or 7.4 (virus production phase) by daily addition of $NaHCO_3$ at 88 g l^{-1}. Samples were taken daily to determine cell density, cell infection, virus titer and pH.

All the assays were performed in duplicate.

2.8. 6-well plate cultures

Cells were grown in 6-well plates (Nunc, Denmark); each well contains 4 ml of culture medium. Cells were seeded at density of 2×10^5 cells ml^{-1} and infected at a MOI of 0.005 on day 0. Cells were first grown in MEM + 10% FCS at 37 °C for 24 h; then temperature was shifted to 34 °C and the medium was exchanged to MEM supplemented by the components to be tested. pH of the culture medium was daily checked by visual inspection. The virus was harvested once pronounced cytopathic effect was observed; generally 11–13 days post infection.

2.9. Bioreactor cultures

Cultures were performed in a 7-l bioreactor (BioBraun, Germany) containing 3 l as a working volume, equipped with a pitch blade impeller and a spin filter (pore size: 75 μm) fixed on the axis. During the cell proliferation step, the following conditions were maintained: pH = 7.2 (regulated by CO_2 sparging or addition of $NaHCO_3$ at 88 g l^{-1}), pO_2 at 50% air-saturation by a continuous surface aeration and direct air sparging when required, temperature at 37 °C and agitation rate at 125 rpm.

For sheep pox virus production, pH was maintained at 7.4, pO_2 at 30% air-saturation, agitation rate at 125 rpm and temperature at 34 °C.

Samples were taken daily to determine the following parameters: cell density, cell viability virus titer, glucose, glutamine, lactate and ammonia levels.

2.10. Cell counting

Aliquots of 5 ml of Vero cell culture were washed three times with PBS, then treated in 5 ml of 0.1 M citric acid containing 0.1% crystal violet and 0.1% Triton X-100 and incubated at least for 1 h at 37 °C. The released nuclei were counted using an hemacytometer.

The specific growth rate, μ (h^{-1}) was estimated by the following equation:

$$\mu = \frac{\ln X_n - \ln X_{n-1}}{t_n - t_{n-1}}$$

where X represents the viable cell density per ml, t represents the time points of sampling expressed in hour; the subscripts n and $n - 1$ stand for two succeeding sampling points.

2.11. Virus titration

Sheep pox virus was titrated in microplates on Vero cells as described by Abbas et al. [22]. Briefly serial dilutions (from to 10^{-2} to 10^{-8}) of virus suspension were added to a 96-well plate containing a suspension of Vero cells at a cell density of 1×10^5 cells ml^{-1}. Eight replicate samples were tested for each dilution. After 5 days of incubation at 37 °C and 5% CO_2, the plate was examined under a microscope to enumerate the number of wells exhibiting cytopathic effect (CPE). The number of CPE positive wells was then converted to $TCID_{50}$ titer using the modified Karber method as described by Trabelsi et al. [23]. Virus titer was expressed in $TCID_{50}$ ml^{-1}.

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