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BHK 21 C13 cells for Aujeszky's disease virus production using the multiple harvest process

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Abstract

Production of Aujeszky's disease virus (ADV) from BHK 21 C13 suspension cells using a simple harvest and multiple harvest process mode was examined. We studied growth kinetics of BHK 21 C31 cells in 750 ml spinner flask containing 500 ml of culture medium. In the simple harvest process of ADV production, 425 ml of virus harvest was obtained with a virus titer of $10^{6.4}$ $TCID_{50}$ ml^{-1} which corresponds to 10,676 doses of vaccine. The multiple harvest process resulted in 850 ml of virus harvest with a virus titer of $10^{6.5}$ $TCID_{50}$ ml^{-1} corresponding to 26,877 AD vaccine doses. In conclusion, the multiple harvest process mode using BHK 21 C13 can be considered as a favorable process to produce ADV.

Abbreviations: ADV – Aujeszky's disease virus; AD – Aujeszky's disease; BHK 21 – Baby Hamster Kidney cells; $TCID_{50}$ – Tissue Culture Infectious Dose 50

Introduction

Aujeszky's disease virus (ADV), a member of the *Alphaherpesvirinae*, causes a natural infection in swine. Symptoms and clinical manifestations of infection are dependent on age and the immunological status of the animal, as well as the virulence and exposure dose to the virus (Zuckermann 2000). To reduce financial losses caused by Aujeszky's disease (AD), vaccination of pigs with attenuated live or inactivated vaccines is widely performed. Deletion of genes encoding nonessential glycoproteins in vaccine strains allows serological differentiation between infected and vaccinated animals (Mettenleiter 2000). Such marker vaccines containing a live attenuated virus strain, lacking nonessential glycoproteins, are available for AD control and eradication programs.

Baby Hamster Kidney cells (BHK 21) have been widely used in biotechnological production of therapeutics and veterinary vaccines, such as rabies (Perrin et al. 1995; Kallel et al. 2002) and foot and mouth disease (Radlett et al. 1985).

Laboratory attenuated ADV replicate well in many types of cells e.g., BHK 21 cells (Puentes et al. 1993) or PK-15 cells (Afshar and Dulac 1986; Markuš-Cizelj et al. 1991) which can be used to produce a vaccine against AD. This vaccine can be produced either by traditional cell culture systems, such as roller bottles, spinner flasks or bioreactors.

The aim of this work was to compare a simple harvest and a multiple harvest process mode to produce a vaccine against AD using BHK 21 C13 cells grown in a 750 ml spinner flask containing 500 ml of culture medium.

Materials and methods

Cell line and culture medium

BHK 21 C13 cell line used in this study was obtained from the American Type Culture Collection (CCL 10). The cells were cultured in Glasgow BHK 21 medium (GIBCO, Paisley, Scotland) supplemented with 5% fetal calf serum (FCS) (Sigma, St. Louis, USA). Antibiotics were not used.

Virus strain

gE-ADV (Bartha K-61 strain) multiplied in PK-15 cell culture provided by the Veterina Ltd. (Kalinovica, Croatia) was used throughout this study.

Cell culture conditions

Cells were cultivated in 25–250 cm^2 T-flasks from Corning at initial cell concentration of 5×10^5 cells ml^{-1} with a maximal volume of 200 ml and in 750 ml spinner flasks (TecnoMara, Switzerland) with a maximal volume of 500 ml of culture medium. The agitation rate was maintained at 50 rpm. Cultivations in T-flasks and spinner flasks were carried out at 37 °C in a 5% CO_2 incubator and pH 7.2. Samples were taken daily to determine cell concentration and viability, as well as the concentration of glucose, lactate, glutamine and ammonia. Depending upon the cell concentration, addition of fresh culture medium was carried out every 48 h. During the whole procedure the cells were grown in the suspension without the formation of clumps (Figure 1).

Experimental ADV vaccine preparation

Cells cultivated in spinner flasks were infected with live modified gE-ADV (Bartha K-61 strain).

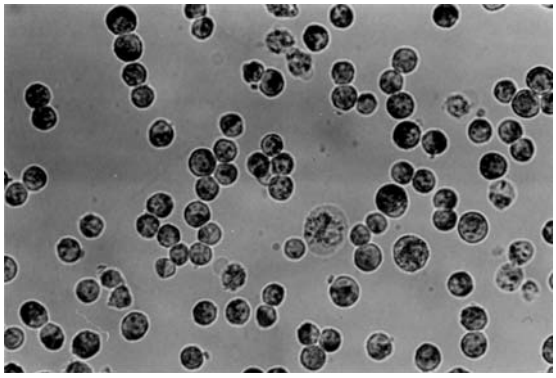

Figure 1. Light microscopy of BHK 21 C13 cells grown in suspension on Glasgow BHK-21 medium + 5% FCS, magnification 400X.

Infected cell supernatants were separated after cell sedimentation, clarified by centrifugation (1000 g for 5 min) and lyophilized after addition of PPGF (Polyvinilpyrrolidone 1% + Peptone 5% + Monosodium Glutamate 1% + Phosphate buffer pH 7.2). Antibiotics were not used during the vaccine production.

Cell counting

Cells were counted in a Fuchs Rosenthal counting chamber using the trypan blue exclusion method. The specific growth rate μ (h^{-1}) was estimated by the following equation: $\mu = (\mathrm{Ln}X_n - \mathrm{Ln}X_{n-1})/(t_n - t_{n-1})$, where X represents the viable cell density per milliliter, t represents the time-points of sampling expressed in hours and the subscripts n and $n\text{-}1$ stand for two successive sampling points.

Metabolite analysis

Glucose was determined using Glucose–PAP colorenzymatic assay kit (Herbos, Sisak, Croatia). Glutamine was determined using Glutamine assay kit GLN-2 (Sigma, St. Louis, USA). Lactate and ammonia were quantified enzymatically by the UV-tests 826-UV and 171-UV, respectively (Sigma, St. Louis, USA).

Virus production

Simple harvest process

BHK 21 C13 cells were infected by the gE-Bartha K-61 strain of ADV at a cell concentration of 1×10^6 cells ml^{-1} and an inoculation volume of 1% (v/v) of the culture volume without changing medium. Virus production was performed at 37 °C in 750 ml spinner flasks containing 500 ml of culture medium with a pH of 7.4. The agitation rate was set to 50 rpm. Samples were taken daily to determine cell concentration and viability. Virus titer was determined by the microtitration method (Lojkić et al. 1992) using secondary cell cultures of chicken fibroblasts (KF/1). Virus titer was estimated by the Spearman–Kärber method and expressed in $TCID_{50}$ ml^{-1}. All assays were performed in duplicate.

Multiple harvest process

BHK 21 C13 cells were infected by the gE-Bartha K-61 strain of ADV at a cell concentration of 1.1×10^6 cells ml^{-1} and an inoculation volume of 1% (v/v) of the culture volume without changing medium. Virus production was performed at 37 °C in 750 ml spinner flasks containing 500 ml of culture medium. The agitation rate was set to 50 rpm. 48 h post infection the agitation was stopped and about 90% of the culture medium was replaced with fresh medium. Before medium change, the agitation was stopped and the cells were allowed to sediment for 5 h. During that time more than 90% of cells sedimented down to the bottom of the spinner flask. The same procedure was repeated at 96 and 168 h post infection. Samples were taken daily for cell counting and for determination of the virus titer. Virus titration was performed in duplicate.

Virus titration

Tenfold virus dilutions were prepared using Glasgow BHK 21 medium (GIBCO, Paisley, Scotland) with 1% FCS (Sigma, St. Louis, USA) as diluents and 0.02 ml of each dilution was inoculated onto chicken embryo fibroblast cultures in microtiter plates (eight wells per dilution). The number of wells infected after 3, 5, and 7 days was recorded, and the 50% tissue culture infective dose per ml was calculated by the method of Spearman–Kärber. All assays were performed in duplicate.

Results and discussion

Cell culture growth

Growth kinetics of BHK 21 C13 cells (Figure 1) are well established and a typical growth curve is presented in Figure 2. Cultures were seeded with 5×10^5 cells ml^{-1} in 10 ml of culture medium and maintained for 10 days. To avoid nutrient limitation cells were diluted by addition of fresh culture medium every 48 h at proportion indicated in Table 1. At day 10, cell density and cell viability equaled 1.1×10^6 cells ml^{-1} and 97%, respectively. The specific growth rate in T-flasks ranged from 0.01 to 0.048 h^{-1} (mean 0.022 h^{-1}) and for spinner flasks from 0.01 to 0.018 h^{-1} (mean 0.015 h^{-1}). The average specific growth rate of BHK 21 C13 cells during exponential growth was 0.048 h^{-1}, corresponding to an average doubling time of 14.5 h. These results are in agreement with the data reported for BHK-21 grown in spinner flasks in serum-free media (Kallel et al. 2002) or BHK-Ankara 66 strain (Gümüşdereliouğlu et al. 2001).

Glucose and lactate were measured during the cultivation period (Table 1). As can be seen glucose and lactate levels changed markedly over the first 48 h. At the end of the cultivation period glucose level was 11.4 mM while lactate level was equal to 12.3 mM and glutamine and ammonia concentrations were equal to 0.14 and 1.96 mM, respectively. Lactate was below inhibitory level of 22 mM as reported for stirred BHK 21A culture (Cruz et al. 2000) while ammonium was in the concentration range which already tends to a growth reduction of BHK cells grown on microcarriers (Butler and Spier 1984).

Virus production

Simple harvest process

To study ADV production, BHK 21 C13 cells were grown in spinner flasks containing 500 ml of culture medium. Once the cell density level reached 1×10^6 cells ml^{-1} 5.0 ml of gE-Bartha K-61 strain of ADV suspension ($10^{5.9}$ $TCID_{50}$ ml^{-1}) were

added to the cell culture (MOI 0.01). Figure 3 indicates that after ADV inoculation, cell density started to decrease. At one-day post infection there was 92% of viable cells, whereas at the end of process only 45% of the cells were still viable. Virus harvest obtained after 96 h post infection, yielded 425 ml and virus titer calculated using the Spearman–Kärber formula, was equal to $10^{6.4}$ $TCID_{50}$ ml^{-1} . Since one AD vaccine dose requires $10^{5.0 \pm 0.5}$ $TCID_{50}$ ml^{-1} (Veterina Ltd., Kalinovica, Croatia – Technical documentation) 10,676 doses could be prepared during this experiment.

Multiple harvest process

To improve the traditional approach of ADV production, we studied the multiple harvest process mode to produce a vaccine that fulfils the standard quality requirements.

BHK 21 C13 cells were cultivated in a spinner flask containing 500 ml of culture medium. 1.1×10^6 cells ml^{-1} were inoculated with 5 ml of gE-Bartha K-61 strain (MOI 0.01) as for the simple harvest procedure. The spinner flask was agitated at 50 rpm during 168 h. Figure 4 shows cell number evolution of viable and nonviable cells during the multiple harvest process. 48 h post infection cell viability was equal to 88% (Figure 4). The agitation was stopped and 450 ml of virus harvest was obtained with an average virus titer of $10^{6.4}$ $TCID_{50}$ ml^{-1} (1st harvest). At the same time 450 ml of fresh medium was added into the spinner flask and the incubation was performed for further 48 h. 96 h post infection (35% of viable cells) 400 ml of virus harvest was obtained with a virus titer of $10^{6.6}$ $TCID_{50}$ ml^{-1} (2nd harvest). An equivalent volume of fresh medium was added and incubation was performed for further 72 h. 168 h post infection (2% of viable

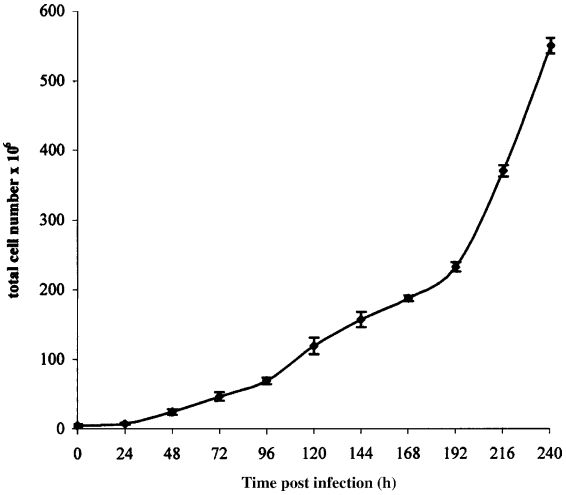

Figure 2. Growth of BHK 21 C13 cells in a spinner flask containing 500 ml of culture medium.

Table 1. Cell growth, glucose/glutamine consumption and lactate/ammonia production for BHK 21 C13 cells during cell amplification in a spinner flask

Time (h)	Culture medium volume(ml)	Total cell number ($\times 10^6$)	Glucose (mM)	Lactate (mM)	Glutamine (mM)	Ammonia (mM)
0	10	5.0	24.4 ± 0.50	1.7 ± 0.04	1.95 ± 0.21	0.09 ± 0.02
24	10	7.5	17.2 ± 0.65	7.1 ± 0.25	0.70 ± 0.16	1.20 ± 0.08
48*%400	10	24.2	14.7 ± 0.07	12.9 ± 0.42	0.20 ± 0.13	2.00 ± 0.25
	50	24.2	20.1 ± 1.23	3.8 ± 0.26	1.00 ± 0.23	0.80 ± 0.06
72	50	46.0	17.9 ± 0.80	6.0 ± 0.42	0.12 ± 0.08	1.65 ± 0.43
96*%140	50	68.5	15.5 ± 1.05	13.3 ± 0.58	0.11 ± 0.06	2.00 ± 0.25
	120	68.5	19.3 ± 1.50	7.4 ± 0.55	1.08 ± 0.19	0.11 ± 0.25
120	120	115.2	16.5 ± 0.43	9.5 ± 0.84	0.12 ± 0.08	1.70 ± 0.08
144*%66	120	156.5	12.4 ± 0.95	11.8 ± 0.35	0.06 ± 0.03	1.85 ± 0.41
168**	200	187.2	10.6 ± 0.25	13.5 ± 0.74	0.08 ± 0.05	1.80 ± 0.45
192*%25	200	232.5	8.1 ± 0.37	15.7 ± 0.23	0.02 ± 0.01	2.00 ± 0.08
	250	232.5	12.0 ± 0.56	11.8 ± 0.46	0.42 ± 0.06	1.45 ± 0.28
216*%100	250	370.0	10.0 ± 0.65	14.6 ± 0.42	0.05 ± 0.01	1.95 ± 0.09
	500	370.0	16.5 ± 0.74	8.1 ± 0.07	0.92 ± 0.32	1.05 ± 0.17
240	500	550.0	11.4 ± 0.28	12.3 ± 0.37	0.14 ± 0.04	1.96 ± 0.08

* – Time when fresh medium was added.
** – Time whenT-flasks were replaced with spinner flasks.
% – Percentages indicate the proportion of fresh culture medium added to BHK 21 C13 cells at the indicated times.

cells) 450 ml of virus harvest were obtained with a virus titer of $10^{3.6}$ $TCID_{50}$ ml^{-1} (3rd harvest). At the 3rd harvest, a considerable decrease in cell viability and virus titer was observed. On the basis of these results and including only the 1st and 2nd harvest, a total virus harvest of 850 ml was obtained with an average virus titer of $10^{6.5}$ $TCID_{50}$ ml^{-1}. Therefore, using the multiple harvest process, it is possible to produce 26,877 doses of AD vaccine.

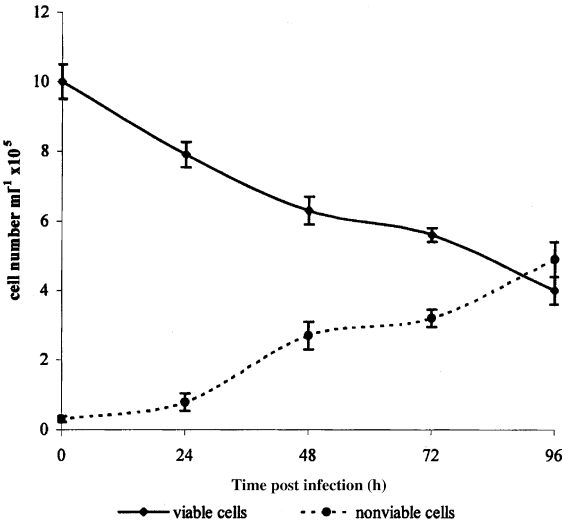

Figure 3 Evolution of viable/nonviable BHK 21 C13 cells during simple harvest production process of ADV.

The comparison between the simple harvest and the multiple harvest processes of ADV production is summarized in Table 2. As indicated, the multiple harvest process resulted in a 2.52 times higher total number of vaccine doses while process duration was similar. During the first and the second harvest the protective activity of inactivated supernatant has been compared to that produced and prepared with the roller bottles using the PK-15 cells (data not shown). This comparison has shown that the ADV produced with the multiple harvest process can be used for the preparation of vaccines with the same protective activity as vaccines produced with the roller bottles process.

The Aujeszky vaccine is routinely produced by Smith Kline-RIT by microcarrier technology using swine testicular cell line (NLST) in 150 l fermentor (Baijot et al. 1987). This production resulted in 2x higher cell density and virus titer expressed as Elisa antigen unit than when using the stationary culture process. This improvement can be explained by a better process regulation related to pH and pO_2 control. Today most of virus production processes are based on serum containing medium. To overcome the disadvantages associated with the use of serum, different serum-free or protein-free media were developed for cultivation of cell lines or virus production like influenza virus (Merten et al. 1996), poliovirus (Merten et al. 1997) and rabies virus (Perrin et al. 1995; Kallel et al. 2002). This approach could be considered in our further

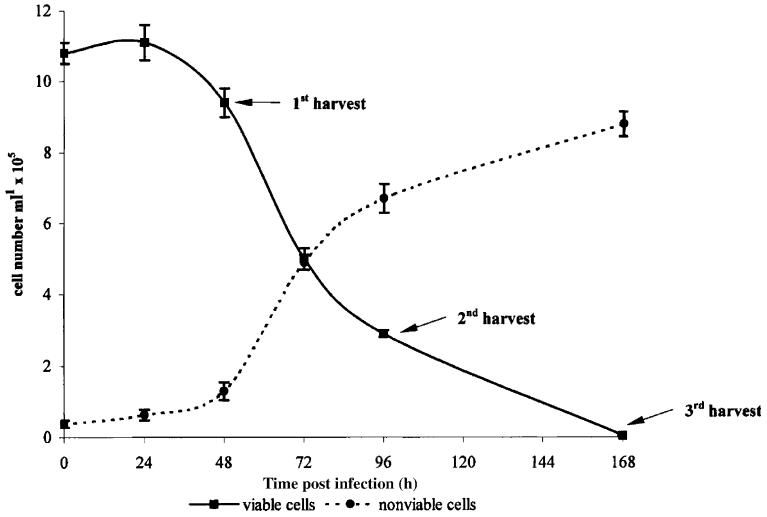

Figure 4. Evolution of viable/nonviable BHK 21 C13 cells during multiple harvest production process of ADV.

Table 2. Comparison of simple harvest and multiple harvest process of BHK 21 C13 biomass utilization during ADV production

Process	Harvest volume	Virus titer ($TCID_{50}ml^{-1}$)	Vaccine doses	Post infection time(h)
Simple harvest	425	$10^{6.4}$	10676	96
Multiple harvest	850	$10^{6.5}$	26877	96

experiments of the ADV production since a serum-free medium which supported cell growth of the BHK21/BRS cells in T- and spinner flasks was developed (Merten et al. 1999).

Conclusion

This study indicates that multiple harvest process is easily performed and is suitable for AD vaccine production compared to simple harvest process, the number of produced doses is increased by 2.52 times.

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