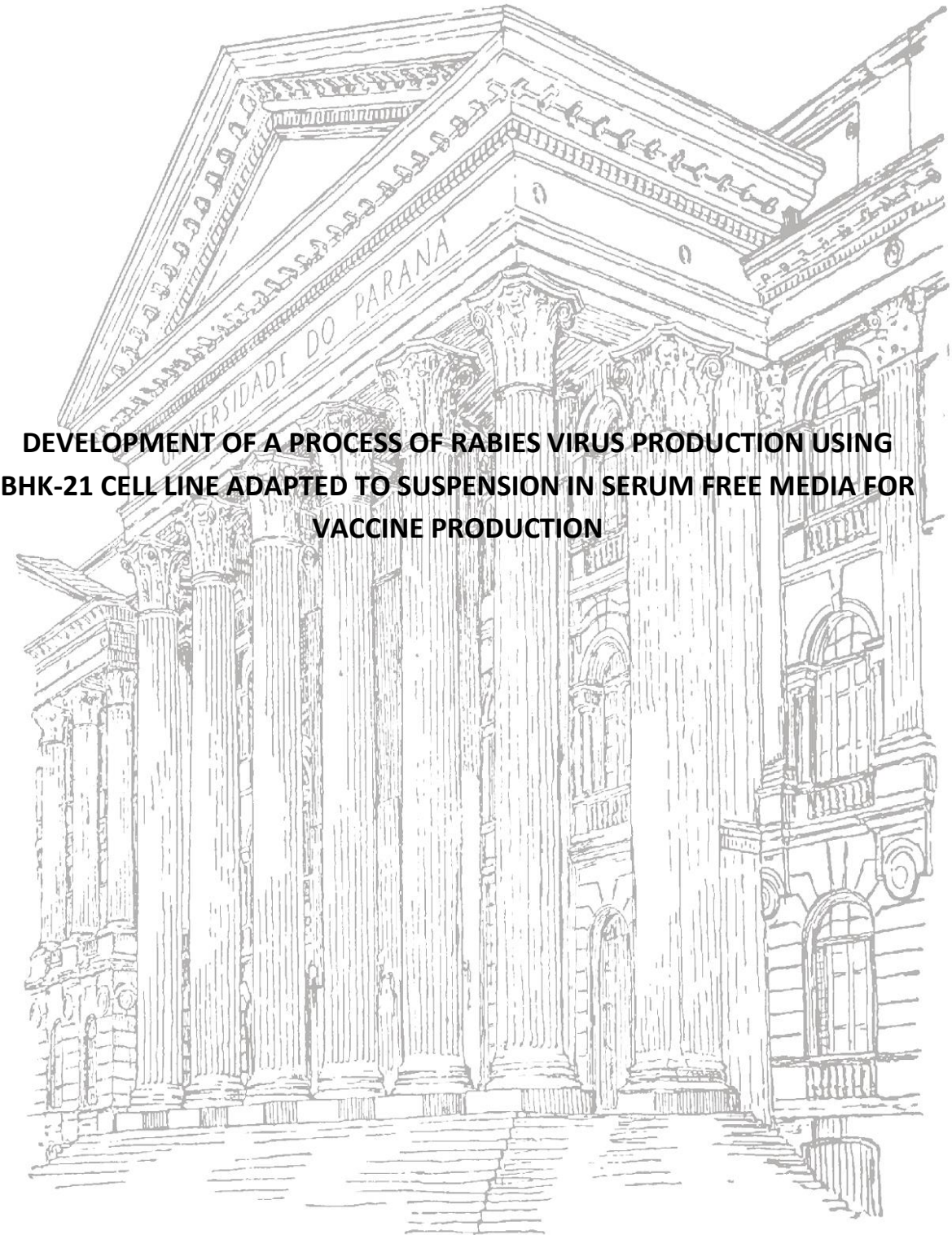
UNIVERSIDADE FEDERAL DO PARANÁ

RAQUEL KOEHLER SANSON



DEVELOPMENT OF A PROCESS OF RABIES VIRUS PRODUCTION USING BHK-21 CELL LINE ADAPTED TO SUSPENSION IN SERUM FREE MEDIA FOR VACCINE PRODUCTION

CURITIBA

2013

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DEVELOPMENT OF A PROCESS OF RABIES VIRUS PRODUCTION USING BHK-21 CELL LINE ADAPTED TO SUSPENSION IN SERUM FREE MEDIA FOR VACCINE PRODUCTION

Dissertação apresentada ao Programa de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, Setor de Tecnologia da Universidade Federal do Paraná, como requisito parcial para a obtenção do título de Mestre em Engenharia de Bioprocessos e Biotecnologia.

Orientador: Prof. Dr. Carlos Ricardo Soccol

Co-orientador: Prof[a]. Dr[a].Vanete Thomaz Soccol

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UNIVERSIDADE FEDERAL DO PARANÁ
Programa de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia
Setor de Tecnologia

UFPR

RELATÓRIO DE DEFESA DE DISSERTAÇÃO DE MESTRADO

Ao primeiro dia do mês de outubro de 2013, na Sala de Aula do PPGEBB, por Videoconferência, no primeiro andar das Usinas Piloto B, no Centro Politécnico da Universidade Federal do Paraná, Jardim das Américas, foi instalada pela Profª Drª Luciana Porto de Souza Vandenberghe, Coordenadora do Curso de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, a banca examinadora para a Septuagésima Nona Defesa de Dissertação de Mestrado, área de concentração: Saúde Humana e Animal. Estiveram presentes no Ato, além da Coordenadora do Curso de Pós–Graduação, professores, alunos e visitantes.

A Banca Examinadora, atendendo determinação do Colegiado do Curso de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia ficou constituída pelos membros: Prof Dr Craic Faulds (Aix Marselle Université), Prof Dr Jean-Claude Sigoillot (Aix Marselle Université), Profª Drª Vanete Thomaz Soccol (UFPR), e Prof Dr Carlos Ricardo Soccol (UFPR- orientador da dissertação).

Às 9h00, a banca iniciou os trabalhos, convidando a candidata **Raquel Koehler Sanson** a fazer a apresentação da dissertação intitulada: **"Development of a Processo of Rabies Virus Production Using BHK-21 Cell Lines Adapted to Suspension in Serum Free Media for Vaccine Production"**. Encerrada a apresentação, iniciou-se a fase de arguição pelos membros participantes.

Tendo em vista a dissertação e a arguição, a banca composta pelos membros Dr Craic Faulds, Dr Jean-Claude Sigoillot, Drª Vanete Thomaz Soccol, e Dr Carlos Ricardo Soccol declarou a candidata Aprovada (de acordo com a determinação dos Artigos 59 a 68 da Resolução 65/09 de 30.10.09).

Curitiba, 1º de Outubro de 2013.

Profª Drª Vanete Thomaz Soccol

Prof Dr Carlos Ricardo Soccol

Profª Drª Luciana Porto de Souza Vandenberghe

ACKNOWLEDGMENTS

I would like to express my gratitude to my supervisors Professor Carlos Ricardo Soccol and Professor Vanete Thomas Soccol, for the useful comments, remarks and engagement through the learning process of this master thesis.

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I would like to thank my family, for unconditional love, understanding my absence and supporting me in difficult time.

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And mainly, I thank God for everything I have and for what I am.

RESUMO

Soro animal é usado em cultivo celular por causa de seus fatores nutricionais, porém, seu uso é um risco potencial à saúde, devido a possível presença de agentes adventícios, tais como vírus e príons, e também é um dos principais responsáveis por reações alérgicas em cães. Células BHK-21 adaptadas à suspensão crescendo em meio de cultura padrão com 3% de soro fetal bovino foram submetidas à adaptação em meio de cultura livre de soro. Os meios de cultura livre de soro usados neste estudo foram VP-SFM, Ex-Cell 302 e Cellvento BHK-200. As células foram adaptadas ao VP por trocada direta do meio de cultura, ao Ex-Cell por troca direta e gradual do meio. Adaptação a Cellvento só foi possível usando células previamente adaptada a VP-SFM. As células adaptadas ao meio de cultura Ex Cell 302 apresentaram melhores resultados de crescimento. Todas as células adaptadas são capazes de produzir vírus da raiva. Porém, células adaptadas em VP-SFM apresentaram a melhor produtividade viral. Entretanto, produção de vírus utilizando Cellvento BHK-200 apresenta o melhor potencial econômico.

Palavras chaves: vacina antirrábica veterinária, meio de cultura livre de soro, BHK-21, adaptação celular.

ABSTRACT

Animal serum is used in cell culture because of its nutritional factors, however, their use not only is a potential risky for health, due to possible presence of adventitious agents, such as virus and príons, but also is the major responsible of allergic reactions in dogs. BHK-21 cells adapted to suspension growing in standard culture media with 3% of fetal bovine serum were submitted to adaptation to serum free culture media. Serum free culture media used in this study were VP-SFM, EX-CELL 302 and Cellvento BHK-200. Cells were adapted to VP by direct media change, to Ex Cell by direct and gradual media change. Adaptation to Cellvento was only possible by using cells priory adapted to VP-SFM. Cells adapted to Ex Cell culture media presented best growth results. All adapted cells have ability to produce rabies virus. Although, cells adapted to VP-SFM presented best virus productivity. However, virus production using Cellvento BHK-200 presents best economic potential.

Keywords: veterinary rabies vaccine, serum free media, BHK-21, cell adaptation.

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LIST OF ABREVIATIONS

BHK	Baby hamster kidney cell line
CHO	Chinese hamster ovary cell line
COS	African green monkey kidney cells (CV1 cells transformed with a defective mutant of SV40)
CVTD	Adaptation to Cellvento BHK 200 media by direct media change
CVTDvp	Adaptation to Cellvento BHK 200 media by direct media change using cells previously adapted to VP-SFM
CVTGvp	Adaptation to Cellvento BHK 200 media by gradual media change using cells previously adapted to VP-SFM
CVTS	Adaptation to Cellvento BHK 200 media by gradual serum reduction
DEAE	Diethylaminoethanol
DMEM	Dulbeco Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
dpi	Day post infection
EXD	Adaptation to Ex cell 302 media by direct media change
EXG	Adaptation to Ex cell 302 media by gradual media change
FBS	Fetal bovine serum
FBS	Fetal bovine serum
FFD_{50}	Fluorescent focus doses 50
FMDV	Foot and mouth disease virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
IgE	Immunoglobulin E
MDCK	Madin-Darby canine kidney cell line
MDSS2	Serum free medium developed by Merten et al.[20], 1994.
MOI	Multiplicity of infection
NIL2	Embryo hamster cell line
Panaftosa	Pan American Centre for Foot-and-Mouth Disease
PBS	Phosphate buffered saline
PV	Rabies Pasteur Virus
SCM	Standard culture medium, comprising DMEM/Ham's F12 1:1 supplemented with 3% of FBS
SCMI	Standard culture medium for infection, comprising DMEM/Ham's F12 1:1 supplemented with 1% of FBS.
SFM	Serum free medium
Tecpar	Technology Institute of Paraná
UFMG	Federal University of Minas Gerais
UFPR	Federal University of Paraná
VPD	Adaptation to VP-SFM media by direct media change
VPG	Adaptation to VP-SFM media by gradual media change
VP-SFM	Commercial serum free medium developed for human viral vaccine production
WHO	World Health Organization

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PRESENTATION

Federal University of Paraná (UFPR) is the oldest University in Brazil. UFPR is reference in higher education for the Paraná State and for Brazil, because of importance and Excellence of graduation, undergraduate, specialization, master and doctoral courses, which are guided by the principle of indivisibility of Teaching, Research and Extension.

The Department of Bioprocess Engineer and Biotechnology of UFPR, through its graduation and undergraduate courses (master, doctoral and post-doctoral) has strong international integration, as well as strong partnerships with universities and research institutes in several countries. The course has among its specific objectives, qualify technical professionals and managers with skills and competencies to participate actively and interdisciplinary in activities of industrial biotechnology. These professionals are specialized in research and development of new products, especially in areas of Agribusiness and Agrifood Biotechnology and Human and Animal Health. In the field of human and veterinary health, this department has developed different projects in partnership with Tecpar.

Technology Institute of Paraná (Tecpar) is a government company that performs research, development and innovation. In the field of industrial biotechnology plays an important role on the public health, as supplier of veterinary rabies vaccine for government mass vaccination. Besides, produces tetanus protein to be conjugated to bacterial triple vaccine, kits for diagnostic of zoonosis, and more recently, kits for detection of HIV and HCV in transfusional blood.

Rabies vaccine laboratory has had important role in rabies prophylaxis in Brazil. In decade of 1970, Tecpar started large scale production of rabies vaccine for human and veterinary use, based on nerve tissue technology, using Fuenzadlida & Palacios methodology, to supply Ministry of Health for vaccination campaigns. Since dog mass vaccination started in Brazil, canine cases reduced 90%. In 2013, Tecpar has received permission from Ministry of Agriculture, Livestock and Supply to produce in large scale rabies vaccine using cell cultivation platform.

1. Introduction

Rabies is an acute encephalitis or meningoencephalitis due to a lyssavirus infection[1]. It is known to be present on all continents except Antarctica. All warm blooded animals are susceptible to rabies. It enters the body through wounds or by direct contact with mucosal surfaces. Rabies virus replicates in the bitten muscle and gains access to motor endplates and motor axons to reach the central nervous system (Figure1)[2,3]. Most of the deaths occur in the absence of post-exposure prophylaxis.

In more than 99% of all human rabies cases, virus is transmitted from dogs. Half of the global human population lives in canine rabies-endemic areas[4]. The most cost-effective strategy for preventing rabies in people is by eliminating it in dogs.

Canine rabies can be eliminated, as it has been demonstrated in North America, Western Europe, Japan and many areas in South America. However, canine rabies is still widespread, occurring in over 80 countries and territories, which are predominantly in the developing world. Mass parenteral vaccination programs remain the mainstay of canine rabies control. Coverage of 70% of dog populations has been sufficient to control canine rabies in several settings[4].

First rabies vaccine was developed by Pasteur in 1885. It was composed of crude suspensions of desiccated, infected brain and spinal cord of rabbit, sheep or goat brain. Occasionally Pasteur's vaccine led to vaccine-induced rabies[5]. Viral inactivation methods using phenol or betapropiolactone had been developed, increasing safety in vaccination, however, nerve-tissue vaccines enhance allergic neuroparalytic accidents caused by encephalitogen associated with myelin in adult mammalian brain, these reactions range from transient to severe paralysis, in some cases resulting in death[5].

A significant reduction in neuroallergic side-effects was obtained with vaccine prepared in suckling mouse brain and inactivated with ultraviolet light or betapropiolactone[6]. It is imperative in the case of mouse suckling that they are under 9 days of age at the time of harvest[5].

After the 1950s, establishment of cell culture technology allowed significant progress in propagation of virus *in vitro* and consequent development of viral vaccines[7]. Rabies vaccines developed and produced by cell culture

technology have demonstrated to be safer and more immunogenic than nerve-tissue vaccines[8].

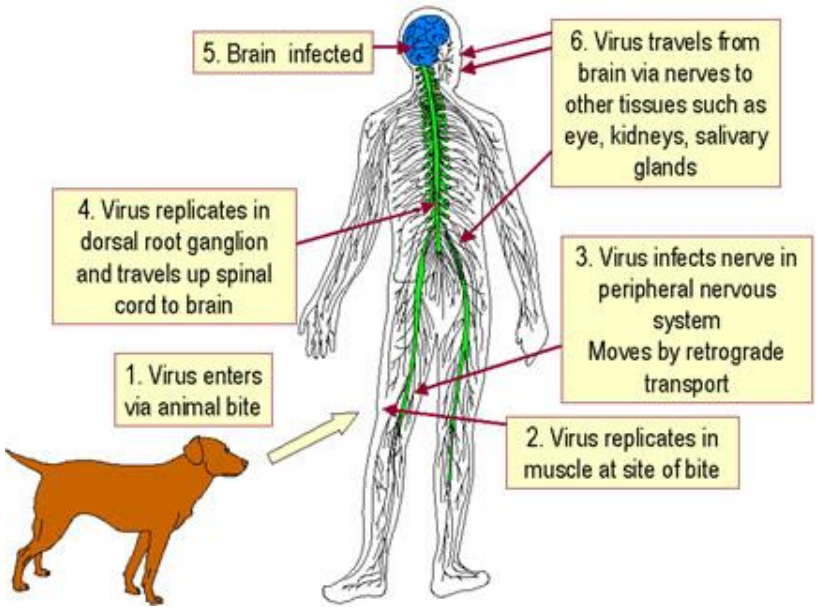

FIGURE 1 – ROUTE OF RABIES VIRUS THROUGH HUMAN BODY.

Source: Hunt, R.[26], 2011.

Since 1984, World Health Organization (WHO) has recommended discontinuation of production and use of nerve-tissue vaccines and their replacement for cell culture vaccines[1]. In Brazil, nerve tissue vaccine was replaced by cell culture vaccine in 2002[1] for human public vaccination and in 2010 for veterinary mass vaccination.

Industrial production of viral vaccines preferably requires use of continuous or immortalized cell lines as the basis for viral multiplication[7]. For human rabies vaccine production, Vero cell line is the most widely used. For veterinary vaccine production cell lines used are baby hamster kidney cell line (BHK-21) or embryo hamster cell line (NIL2) [9-12].

The BHK-21 cell line was isolated from five 1-day-old hamsters[11] and consists of adherent fibroblasts that can also be adapted to suspension culture[13]. Suspension culture is widely used for veterinary vaccine production, due to lower costs of production and large cell quantities attained without need of increasing superficial area[13].

Effective *in vitro* maintenance and growth of animal cells requires culture conditions similar to those found *in vivo* with respect to temperature, oxygen concentrations, pH, osmolality, and nutrients[14].

Culture medium composition is one of the most important factors in culture of animal cells. Its function is to provide appropriate pH and osmolality for cell survival and multiplication, as well as supplying all chemical substances required by the cells that they are unable to synthesize themselves[14].

Some of these substances can be provided by a culture medium consisting of low molecular weight compounds, known as basal media. However, most basal media fail to promote successful cell growth by themselves and require supplementation with more complex and chemically undefined additives such as blood serum[14].

Fetal bovine serum (FBS) is the most frequently employed serum in animal cell culture however calves, horses, and even human sera are also used[14].

FBS is the favorite because it presents low immunoglobulin and high growth factor concentrations. However, it is one of the most expensive components in culture medium[14].

Use of serum in cell culture is important because of nutritional factors, culture-stimulating factors (growth factors, hormones and proteins) and protecting agents, both for biological protection (antitoxin, antioxidant, antiprotease) and for prevention of mechanical damage[14].

However, using animal serum is potential risky for health, due to the possible presence of adventitious agents, such as virus and prions. In addition, serum can contain contaminants such as bacteria, fungi, and mycoplasmas, which can negatively affect cell culture. Another strong limitation to the use of sera is its variability among different lots and suppliers, which hinders the standardization of the culture medium and the reproducibility of culture performance[14].

Furthermore, studies have demonstrated that bovine serum is the major responsible of allergic reactions in dogs[15]. A research was designed to investigate the relationship between IgE reactivity to the vaccines and immediate-type allergic reactions after vaccination in dogs. Sera from 10 dogs that developed immediate-type allergic reactions such as circulatory collapse,

cyanosis, dyspnea, facial edema, and vomiting within 1 hour after vaccination with rabies vaccines and sera from 50 dogs that did not develop allergic reactions after vaccination were collected. Then, IgE reactivity to fetal bovine serum and stabilizer proteins included in the vaccines was measured in sera that had high levels of IgE to the vaccines. Of the eight dogs that developed immediate-type allergic reactions and had high levels of serum specific IgE to the vaccines, seven had specific IgE directed to fetal bovine serum[15].

Later, researchers investigated IgE-reactive components of fetal bovine serum using sera from 16 dogs that exhibited allergic reactions after vaccination. The immunoblot analysis revealed that several fetal bovine serum proteins strongly reacted with IgE in the sera from dogs that showed post-vaccination allergic reactions. The 66-kDa band was detected in the sera from 14 of the 16 dog serum samples analyzed in the immunoblot analysis for fetal bovine serum, and it was speculated to be albumin based on its molecular weight, however, serum IgE reactivity to bovine serum albumin could be detected in only four of the 14 dog samples. These findings demonstrated that a variety of fetal bovine serum components including albumin could function as allergens in dogs that developed allergic reactions after vaccination.

Many attempts have been made to develop culture media that do not need serum supplementation, that is, serum free media (SFM) formulations. One of the greatest difficulties is to design a culture medium that ensures replicate cultures that show stable genetic, metabolic, and kinetic behavior[14].

Despite all the development seen, up to the present time, there is no standard culture medium that could be universally utilized for all cells, since each cell line has a specific metabolic profile and requires an appropriate medium composition[14].

For instance, Cellvento™ BHK-200 medium is a serum free medium, formulated without any animal derived component and optimized for the culture of suspension BHK-21 cells at high-density, viability and efficient propagation of viruses. The medium formulation was developed for the growth and maintenance of BHK-21 suspension cell lines used for viral vaccine production, and qualified for the production of Foot and Mouth Disease virus (FMDV) [27].

Ex Cell® 302 is a serum free medium which has been specifically developed for the long term growth of transformed Chinese Hamster Ovary

(CHO) cells in suspension for the expression of antibodies or protein products. It was developed using only recombinant human proteins that have molecular weights less than 10 kDa[28].

VP-SFM™ is a serum free media expressly designed for virus production, manufactured without any components of animal or human origin. VP-SFM is a ultra-low protein medium designed specifically for the culture of Vero cells. It is also appropriate for the growth of COS-7, MDCK and BHK-21 in suspension. It is particularly suitable for growing viruses[29].

When using a new medium the need for cell adaptation should be considered. The assessment of cellular performance in different culture media is usually challenging and time-consuming, since it requires establishment of the concentration of total and viable cells, over multiple subcultures. In addition it may involve the quantification of residual nutrients, such as glucose and glutamine, of metabolic byproducts, such as lactate, ammonium and alanine, and the concentration of the target product[14].

Cell adaptation is often carried out in static cultures employing tissue culture flasks. However, problems seen at the time of adaptation can be minimized in suspension cultures, since this allows subculture during the log growth phase. Cultures in suspension also allow better medium oxygenation[14] and homogeneity.

Mammalian cells can be adapted to serum free conditions by direct adaptation from serum containing media or gradual weaning. Both procedures require healthy, viable cultures in the mid logarithmic growth phase. During the adaptation phase, growth rates will usually be somewhat slower than growth in media supplemented with serum.

Perrin et al, 1995[20] adapted BHK-21 cells to grow in MDSS2[18] serum free media, for production of an experimental rabies vaccine. Kallel et al, 2002[19] also adapted BHK-21 cell line to grow in different commercial serum free media and evaluated rabies vaccine produced. In both cases, adaptation was carried out with cell growing in monolayer.

2. Aim and objectives

2.1. Aim

To develop a process of production of rabies virus to rabies veterinary vaccine, using BHK-21 cell line in suspension adapted to commercial serum free media.

2.2. Specific objectives

- Adapt BHK-21 cell line to grow in different commercial serum free media;
- Analyze growth kinetics of adapted cells;
- Infect cells with rabies virus and analyze behavior of infected cells;
- Verify virus production capacity of serum free media adapted cells;
- Compare results of adapted cells.

3. Material and methods

3.1. Culture media

Standard culture medium (SCM), comprising DMEM/Ham's F12 1:1 supplemented with 3% of FBS, was used for control cell culture.

Standard culture medium for infection (SCMI), comprising DMEM/Ham's F12 1:1 supplemented with 1% of FBS, was used during virus infection of control cell culture.

The following serum free media VP-SFM™ (Gibco – Invitrogen cell culture), Ex-Cell® 302 (SAFC Biosciences) and Cellvento™ BHK-200 (Merck Millipore) were used. Media were prepared following manufacturer's instructions. No antibiotic was added.

Cryopreservation culture media were prepared using 7% of DMSO, 46.5% serum free media and 46.5% media supernatant of centrifuged cells for cryopreservation.

3.2. Cell line

BHK-21 C13 LVI cell line, adapted to suspension, in passage 118, provided from Tecpar working cell bank, was used. Tecpar BHK-21 C13 LVI cell bank was originated from Panaftosa with 84 passages, cells were adapted to grow at SCM to generate master and working cell bank. Cells were stored at -196°C.

3.3. Virus strain

Rabies Pasteur Virus (PV) supplied from Federal University of Minas Gerais (UFMG) was adapted to grow in BHK-21 C13 in suspension. Adapted virus generate Tecpar master and working virus bank, stored at -80°C. Virus strain was provided from Tecpar working virus bank was used for this study.

3.4. Cell adaptation strategies

Three different cell adaptation protocols were used:

3.4.1. ***Direct medium change (D):*** cell line growing healthily in SCM, with cell density higher than $7.5x10^5$cells/mL, was centrifuged and

resuspended in serum free media at concentration of $2.0x10^5$cells/mL (Figure 2). Subcultures were made until cell adaptation.

3.4.2. ***Gradual medium change (G):*** cell line growing healthily in SCM, with cell density higher than $7.5x10^5$cells/mL, had serum free media added in culture gradually during subculture (Figure 2). Increased quantity of serum free media was only added when cell was growing healthily in previous adaptation phase. In last adaptation step, cells were centrifuged, in order to remove all serum from cultivation, and resuspended in serum free media.

3.4.3. ***Gradual serum reduction (S):*** cell line growing healthily in SCM, with cell density higher than $7.5x10^5$cells/mL, was centrifuged and resuspended in serum free media at concentration of $2.0x10^5$cells/mL. SFM was supplemented with 3% of fetal bovine serum. Serum was decreased from culture gradually during subcultures (Figure 2). Serum was only reduced when cell had normal growth at previous adaptation phase. In last adaptation step, cells were centrifuged, in order to remove all serum from cultivation, and resuspended in serum free media.

During adaptation a control culture in SCM was held to compare growth behavior.

If cells, during adaptation, did not grow as control, half of volume media was replaced (by centrifugation) for fresh medium. This procedure was used to offer better conditions for cell growth (adding nutrients and removing inhibitory metabolites).

Direct media change protocol was used for adaptation of three serum free culture media used in this study. Gradual media change protocol was used to adapt cells to VP-SFM and Ex-Cell 302 serum free media. Gradual serum reduction protocol was used to adapt cells to Cellvento BHK-200 serum free media. Protocols choice followed manufacturer’s recommendations.

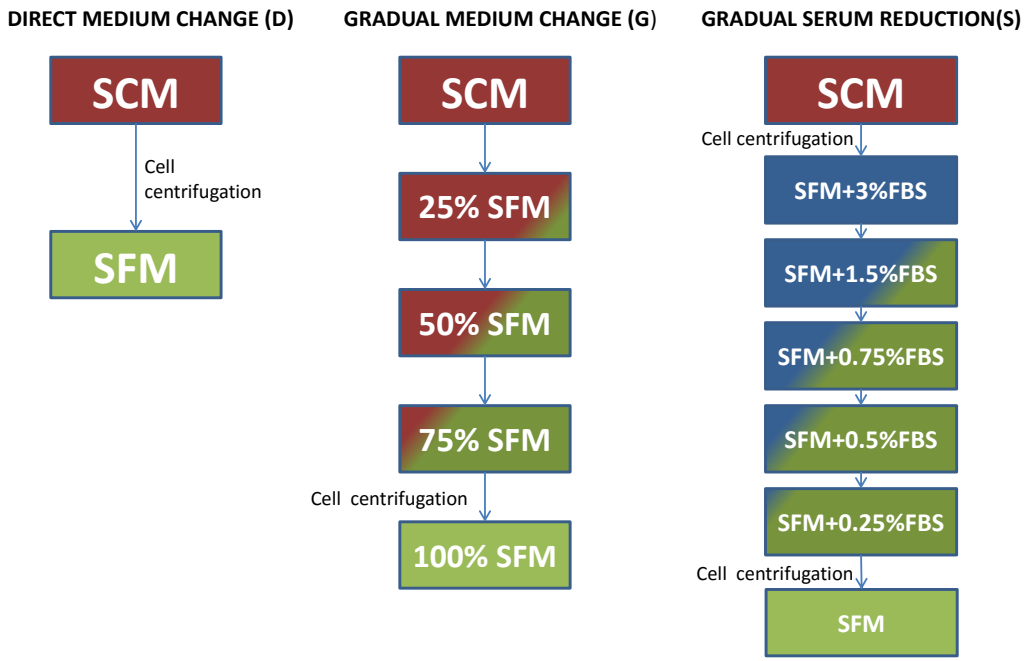

FIGURE 2 – PROTOCOLS USED FOR CELL ADAPTATION TO SERUM FREE MEDIA

3.5. Cell cultivation

Cell cultivation was performed in spinner flasks (Techne, United Kingdom) of 500mL working volume, with 400mL of culture medium, incubated at 37°C and agitated at 55rpm. Innoculum was made with cell density of $2.0x10^5$cells/mL. Subcultures (cells dilution) were made when growth reached $7.5x10^5$cell/mL or higher density.

Sometimes, during adaptation to SFM, even when cells did not reach $7.5x10^5$cells/mL, a subculture was done, usually to try to enhance growth conditions. Also, if cells could not reach cell density higher than $7.5x10^5$cells/mL, innoculum for subculture was increased to $5x10^5$cells/mL, to try making cells reach higher density.

Samples were collected daily for cell count, viability determination, morphology verification, metabolites analysis and pH measurement.

3.6. Cell count

Cell samples were diluted and stained with trypan blue (0.5%) than counted in hemocytometer. Viable cells present intact membrane, and are not colored by trypan blue, not viable cells permit trypan blue to traverse

membrane, being colored by dyer. Total cells, viable cells and cell viability were determined. Morphology, light refringence and size were evaluated.

3.7. Metabolism analysis

Glucose consumption and lactate production were determinate to verify growth limitation by inhibitory levels of lactate or by glucose scarcity.

A dual channel biochemical analyzer YSI 2700 (Yellow Spring Instruments, EE.UU.) was used for glucose and lactate determination. Each channel contains immobilized enzyme on a polycarbonate membrane. One membrane has enzyme glucose oxidase immobilized and catalyzes the following reaction:

$$glucose + O_2 + H_2O \rightarrow gluconic\ acid + H_2O_2 \quad \text{(reaction 1)}$$

In other membrane, the enzyme lactate oxidase catalyzes reaction 2:

$$lactate + O_2 \rightarrow piruvate + H_2O_2 \quad \text{(reaction 2)}$$

Hydrogen peroxide generated independently in each membrane is oxidized in a platinum anode. Electron flow is linearly proportional to concentration of hydrogen peroxide formed, and this, to the concentration of the substance in the sample. Variation of 2% was considered acceptable between successive calibrations.

3.8. Sedimentation tests

Cell lines adapted to serum free media were submitted to sedimentation tests. It consists of taking cell culture, with cell density over than $7.5x10^5$cells/mL, in cold chamber (2-8°C) for 2 or 3 hours until complete cell sedimentation. Carefully, culture media was removed from spinner (about 200 mL) and fresh media was added. This procedure was repeated during 3 consecutive days. Every day, samples were collected to cell count, viability, morphology and metabolic analysis. To be approved in this test, cells should keep their growth behavior and not grow in clumps.

Sedimentation test was performed to simulate industrial rabies virus production where cells have to be sedimented for virus infection. So, adapted cells should be robust enough for this operation.

3.9. Cryopreservation

Seed banks were made with cell lines approved in sedimentation tests. Cryotubes with $1x10^7$cells and 1mL of cryopreservation media were frozen in -80°C freezer. Gradual temperature reduction rate of -1°C/minute was achieved using Mr. Frosty (Nalgene) and isopropyl alcohol. After reaches -80°C, cells were stored at liquid nitrogen (-196°C).

3.10. Growth kinetics

Cell cultivation was performed with 400mL of culture medium at 37°C, in spinner flasks of 500mL working volume (Techne, United Kingdom), agitated at 55rpm, innoculum was made with cell density of $2.5x10^5$. No subculture was done. Daily, samples were collected for cell count, viability determination, morphology verification, metabolites analysis and pH measurement. Cultivation was carried out until cells death.

Specific growth rate, μ (cells/h), was estimated by the following equation[19]:

$$\mu = (LnX_2 - LnX_1)/(t_2 - t_1) \qquad \text{(equation 1)}$$

Where, *X* represents the viable cell density per mL, *t* represents the time points of sampling expressed n hours; the subscripts 1 and 2 stand for two succeeding sampling points.

3.11. Cell infection and virus production

Cell infection was carried out on cultures with cell density of about $1.0x10^6$cells/mL. pH was adjusted to 7.4, with sodium bicarbonate 7.5%, cells were sedimented in cold chamber, 300mL of media was removed. Virus, in multiplicity of infection (MOI) of 0.015, and DEAE (1% of final volume) were added to spinner flask and cells were incubated at 33°C, over intermittent 35

rpm agitation (5 minutes of agitation, 20 minutes stopped) during 90 min. Infection media was added and spinners were again incubated at 33°C over 55rpm.

Daily, samples were collected for cell count, metabolites analysis, pH measurement, virus titer determination and direct immunofluoresce assay. pH was adjusted, to 7.4, cells were sedimented on cold chamber and half volume of media was harvested. Fresh media was added and spinners were again incubated. This procedure was held for 6 days.

3.12. Direct Immunofluorescence *assays*

Infected cells samples (50 µL) were sedimented in microscope slides, fixed with cold 80% acetone and stained with Evans Blue dye (1:40000) and flurescein-labeled antirabies nucleocapsid immunoglobulins (Bio-rad code 357-2114). Slides were analyzed in fluorescence microscope to determine level o cell infection. It was evaluated in terms of fluorescent focus inside the cells (Table 1). This procedure was adapted from Batista and collaborators, 2009[20].

Immunofluorescence assays were conducted to verify cell infection evolution.

TABLE 1 – RELATION BETWEEN INFECTION CLASS AND FLUORESCENCE FOCUSES

Infection class	Fluorescence focus
0	total absence of fluorescent focuses
I	1 to 25% of cells had fluorescence focuses
II	26 to 50% of cells had fluorescence focuses
III	51 to 75% of cells had fluorescence focuses
IV	76 to 100% of cells had fluorescence focuses

3.13. Rabies virus titration

For determination of rabies virus titer [21-23], monolayers of BHK-21 cells on 96 well microtiter plates were infected with sample dilutions and incubated at 37°C with 5% CO_2 for 22h. Cells were then fixed with cold 80% acetone, washed with phosphate buffered saline (PBS) and incubated with flurescein-

labelled antirabies nucleocapsid immunoglobulins (Bio-rad code 357-2114) for 1h at 37°C. The microtiter plates were then observed in fluorescence microscope and titer was expressed as fluorescent focus doses 50 (FFD_{50}) as calculated by Spearman-Kärber method[24].

4. Results and discussion

4.1. Cell adaptation

Table 2 presents strategies used for cell adaptation. Strategies were used following manufacturer's recommendations.

In each adaptation, a control culture with standard culture media (SCM) was carried out to compare results.

TABLE 2 – STRATEGIES USED FOR BHK-21 CELL LINE ADAPTATION TO SERUM FREE MEDIA

Adaptation strategy	Serum free media		
	VP	Ex Cell 302	Cellvento BHK-200
Direct media change (D)	X	X	X
Gradual media change (G)	X	X	
Gradual serum reduction (S)			X

4.1.1. Adaptation to VP-SFM from SCM

- **Control culture**

Control culture presented a standard behavior (Figures 3A and 3B) during subcultures. Cell morphology, size and refringence were normal. Cell density reached $7.5x10^5$/mL or higher after two days of cultivation, cell viability was higher than 80%, usually, cells doubled or tripled every day. Glucose consumption, lactate production and pH values were very similar during subcultures.

- **Direct Medium Change**

In adaptation using direct media change from SCM to VP (VPD), during 18 days and 10 subcultures, cells presented abnormal morphology, size and growth (Figure 4A). In some subcultures, cells have grown in clumps. There was much cell debris in medium and viability was very low, reaching 18% in fourth adaption day. In this period, between some subcultures, cells were centrifuged and half of culture media was replaced, in order to remove inhibitory metabolites, adjust pH and supply nutrients. In eleventh subculture, cells started to present similar behavior to control cells, but in next subculture (12^{nd}), cells

formed aggregates, so 3 more subcultures were carried out, to confirm cells behavior (Figures 4A and 4B). As cells maintained behavior, and clumps were not formed anymore, cells were submitted to sedimentation test. During sedimentation test, no clump was formed and culture reached density of $1.8x10^6$ cells/mL. Cells were considered adapted to VP-SFM culture media and robust enough to virus production. Seed bank was made.

Adaptation to VP-SFM took 26 days and 15 passages.

- **Gradual Medium Change**

In adaptation using gradual media change from SCM to VP (VPG), first step of media change was 75% of SCM and 25% of VP, no alteration in cell behavior or metabolites was observed (Figures 5A and 5B), after 3 subcultures, a new step of media substitution was done, 50% of SCM and 50% of VP. No alteration in cell behavior or metabolites was observed. Next step was 25% of SCM and 75% of VP. In this step, cell viability and cell growth decreased during third and fourth subcultures, but in next passage, cell growth behavior became normal and cell viability improved in sixth subculture. So next media change step was done: 0% of SCM and 100% of VP. In this adaptation stage, cells morphology was abnormal, clumps were formed, and much cells debris was observed in media. In some subcultures, cells were centrifuged and half of culture media was replaced in order to remove inhibitory metabolites, adjust pH and supply nutrients. Even though, no improvement in cells morphology was observed, and also, glucose consumption and lactate production were higher than during prior adaptation steps and control cells. As adaptation in direct media change was reached. This adaptation procedure was stopped, and cells were not considered adapted.

Adaptation procedure took 49 days and was unsuccessful.

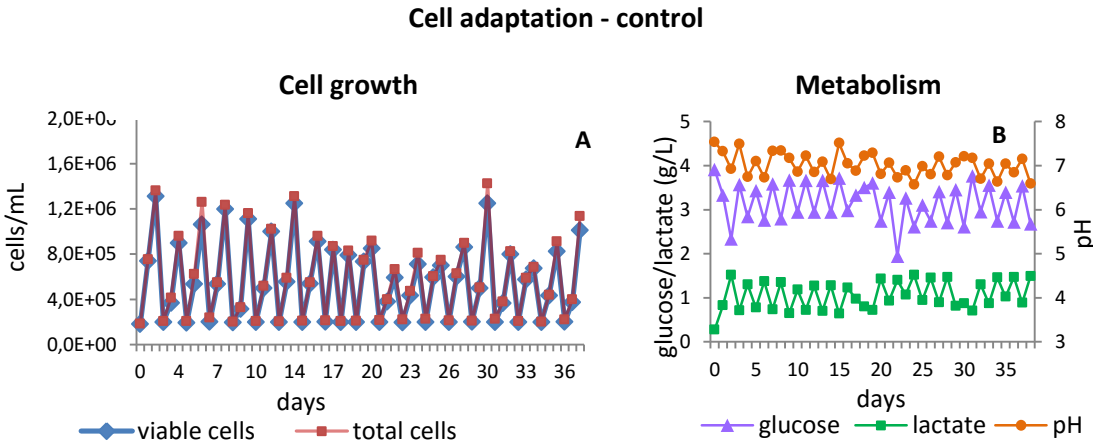

FIGURE 3 – ADAPTATION TO VP SERUM FREE MEDIA: CONTROL CULTIVATION.
Growth (A) and metabolites (B) behavior of control BHK-21 cell line, in SCM. Cultivation was carried out during adaptation of BHK-21 cell line to VP serum free media.

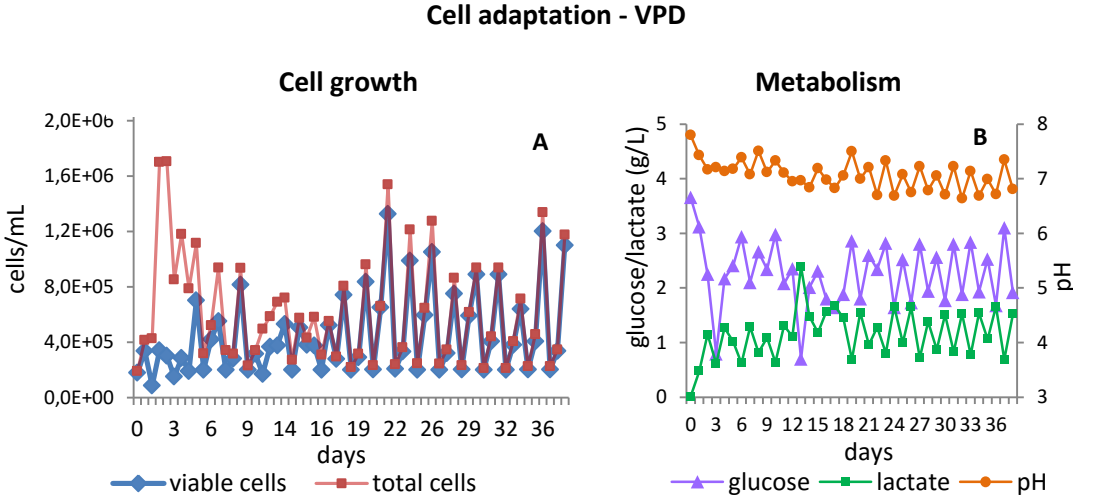

FIGURE 4 – ADAPTATION TO VP SERUM FREE MEDIA BY DIRECT MEDIA CHANGE.
Growth (A) and metabolites (B) behavior of BHK-21 cell line, during adaptation to VP-SFM, by direct media change.

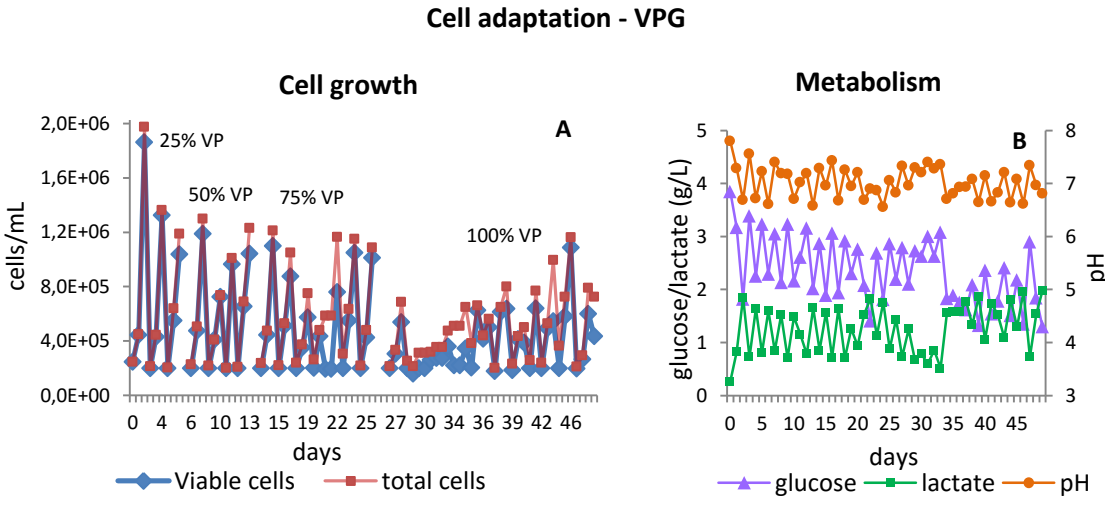

FIGURE 5 – ADAPTATION TO VP SERUM FREE MEDIA BY GRADUAL MEDIA CHANGE.
Growth (A) and metabolites (B) behavior of BHK-21 cell line, during adaptation to VP-SFM, by gradual media change.

4.1.2. Adaptation to Ex Cell 302 from SCM

- **Control culture**

Control culture presented standard behavior (Figures 6A and 6B) during subcultures. Cell morphology, size and refringence were normal. Cell density reached $7.5x10^5$ or higher after two days of cultivation, cell viability was higher than 80%, cells doubled or tripled every day. Glucose consumption, lactate production and pH values were very similar during subcultures.

- **Direct Medium Change**

In adaptation using direct media change from SCM to Ex Cell 302 (ExD), during 26 days and 10 subcultures, cells presented abnormal morphology, size and growth (Figure 7A). There was much cell debris in medium but cell viability had not decreased as much as in VPD adaptation, lowest viability value was 72%. In this period, between some subcultures, cells were centrifuged and half of culture media was replaced. In eleventh subculture, cells started to present similar growth and metabolites behavior to control culture. Three more subcultures were done, to confirm cells behavior (Figures 7A and 7B). As cells maintained behavior, they were submitted to sedimentation test. During sedimentation test, no clump was formed and culture reached density of $1.2x10^6$cells/mL. Cells were considered adapted to Ex Cell 302 culture media and robust enough to process of virus production. Seed bank was made.

Adaptation to Ex Cell 302 by direct media change took 36 days and 14 passages.

- **Gradual Medium Change**

In adaptation using gradual media change from SCM to Ex Cell 302 (ExG), first step of media change was 75% of SCM and 25% of Ex Cell 302, no alteration in cell growth behavior nor in metabolism was observed (Figures 8A and 8B), but cell morphology and size were abnormal and media had much cells debris. Even though, after 4 subcultures, a new step of media substitution was done, 50% of SCM and 50% of Ex Cell 302. No alteration in cell behavior or metabolites was observed, cell morphology and size became normal and cells debris disappeared. Next step was 25% of SCM and 75% of Ex Cell 302.

In this step, 4 subcultures were done, cell growth behavior was normal, glucose consumption was higher, but can be explained due to higher cell density. So next media change step was done: 0% of SCM and 100% of Ex Cell 302. In this adaptation stage, no problem was observed. Morphology, growth and metabolites had same behavior that control cells. So, cells were submitted to sedimentation test: no clump was formed and culture reached density of $1.3x10^6$cells/mL. Cells were considered adapted to Ex Cell 302 culture media and robust enough to virus production. Seed bank was made.

Adaptation to Ex Cell 302 by gradual media change took 36 days and 18 passages. ExG adaptation took same time than ExD adaptation, but more subcultures were done, this is due to in ExD adaptation, cells took longer to reach cell density higher than $7.5x10^5$.

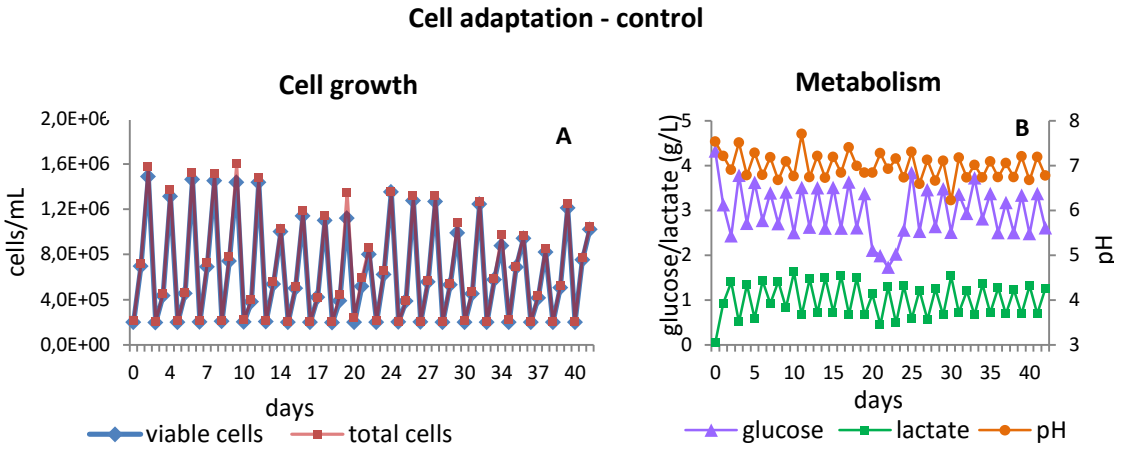

FIGURE 6 – ADAPTATION TO EX CELL 302 SERUM FREE MEDIA: CONTROL CULTIVATION.
Growth (A) and metabolites (B) behavior of control BHK-21 cell line, in SCM. Cultivation was carried out during adaptation of BHK-21 cell line to Ex Cell 302 serum free media.

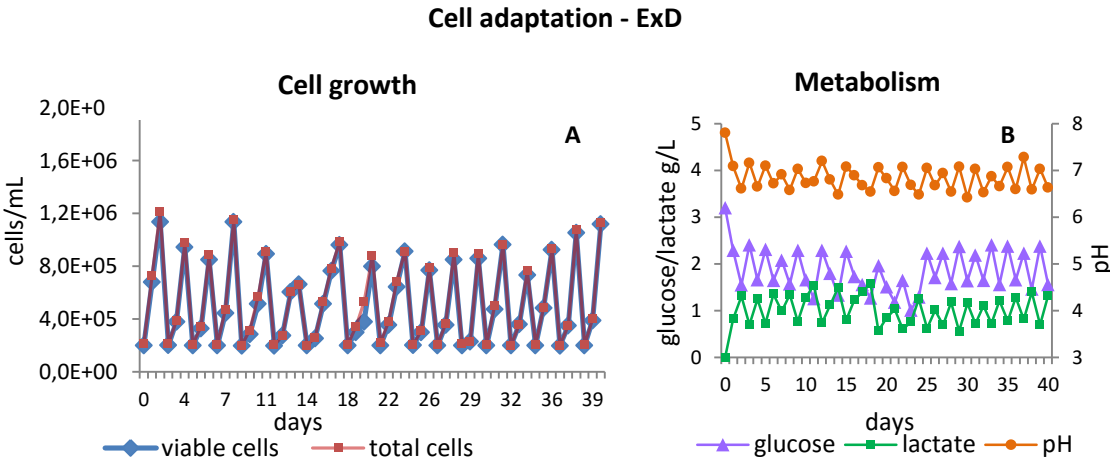

FIGURE 7 – ADAPTATION TO EX CELL 302 SERUM FREE MEDIA BY DIRECT MEDIA CHANGE.
Growth (A) and metabolites (B) behavior of BHK-21 cell line, during adaptation to Ex Cell 302 serum free media, by direct media change.

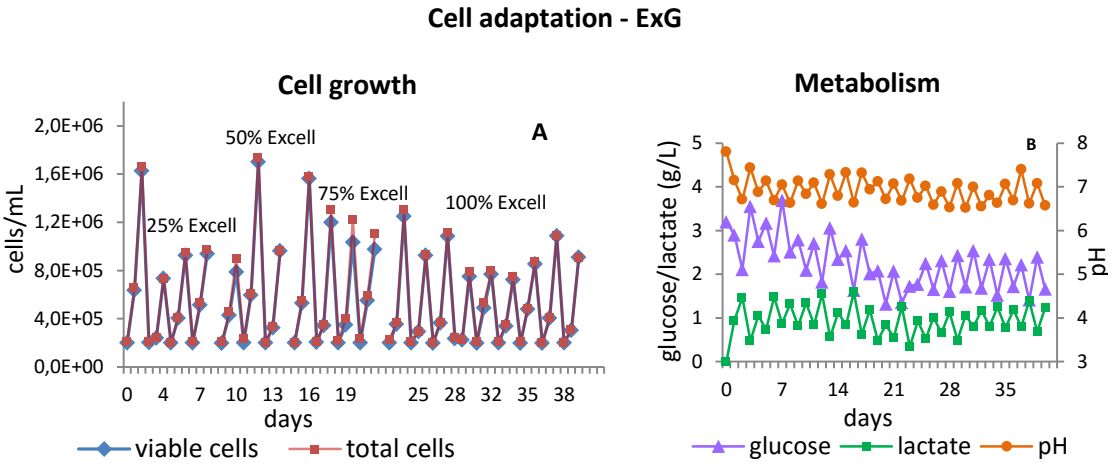

FIGURE 8 – ADAPTATION TO EX CELL 302 SERUM FREE MEDIA BY GRADUAL MEDIA CHANGE.
Growth (A) and metabolites (B) behavior of BHK-21 cell line, during adaptation to Ex Cell 302 serum free media, by gradual media change.

4.1.3. Adaptation to Cellvento BHK-200 from SCM

- **Control culture**

Control culture presented standard behavior (Figures 9A and 9B) during subcultures. Cell morphology, size and refringence were normal. Cell density reached $7.5x10^5$ or higher after two days of cultivation, cell viability was higher than 80%, cells doubled or tripled every day. In some subculture cells quadrupled in 24 hours. Glucose consumption, lactate production and pH values were very similar during subcultures.

- **Direct Medium Change**

In adaptation using direct media change from SCM to Cellvento BHK-200 (CVTD), during 50 days and 15 subcultures, cells presented abnormal morphology, size and growth (Figures 10A and 10B). In some subcultures, cells have grown in clumps. There was much cell debris in medium. In this period, between some subcultures, cells were centrifuged and half of culture media was replaced, in order to remove inhibitory metabolites, adjust pH and supply nutrients. Even though, in no subculture, cells reached higher density than $5.8x10^5$cells/mL. No enhance in cell morphology or growth were observed with time, so this adaptation work was stopped without any cell adaptation.

- **Gradual Serum Reduction**

In adaptation using gradual serum reduction from SCM to Cellvento BHK-200 (CVTS), first step was to centrifuge cells from SCM and resuspend cells in Celvento with 3% of FBS. In this step of adaptation, cells presented normal morphology, growth and metabolism (Figures 11A and 11B). Four subcultures were performed and first step of serum reduction was done (reduction to 1.5%). Cells also have presented normal morphology, growth and metabolism. So, after 3 subcultures, a new adaptation step was done: Cellvento supplemented with 0.75% of FBS. This step presented no problem with cell morphology, cell growth, or metabolism. Next step of serum reduction was carried out (Cellvento with 0.5% of FBS), but contaminated and had to be discharged (results not shown). A new subculture was done with Cellvento added with 0.5% of FBS.

This step had not showed any cultivation problem. Cell growth, morphology and metabolites were similar to standard.

In next step, Cellvento supplemented with 0.25% of FBS, cells started to show abnormal growth, in some subcultures, even with media replacement, they could not reach density of $7.5x10^5$cell/mL. Cell innoculum was increased to $5.0x10^5$ cells/mL but cells could not double. Cell morphology became very irregular; media had much cells debris and different sizes of cells in culture.

After 41 passages and 116 days trying to adapt cells work was stopped, without cell adaptation.

As Cellvento BHK-200 is a culture media developed to be used in veterinary vaccine production, its price is about 10 times cheaper than other serum free media used in this study. Adaptation of BHK-21 to this media has a very important economic perspective. So failure in adaptation using previous strategies leads to try a new strategy. In this new strategy, cells already adapted to VP serum free media were used to adapt to Cellvento BHK-200. Two adaptation protocols were used direct media change and gradual media change. Below are presented results of this new protocol.

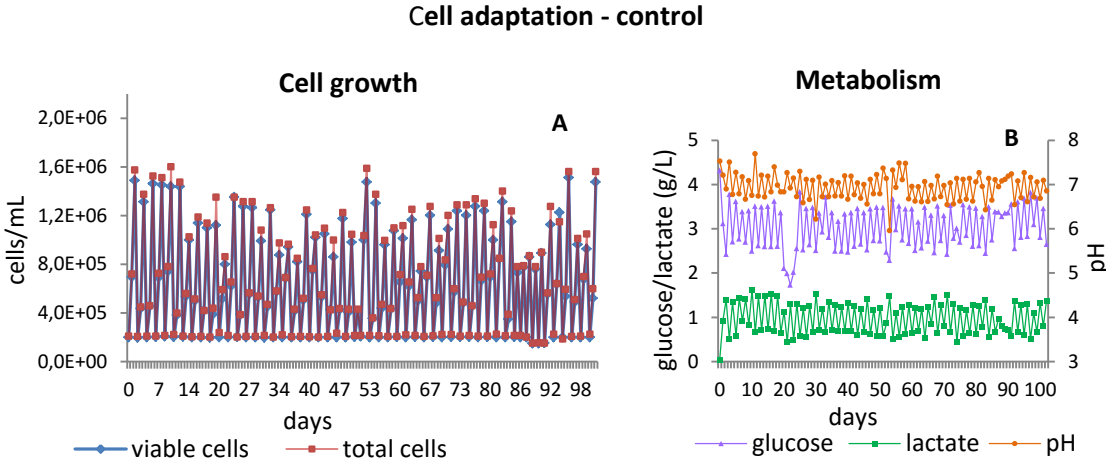

FIGURE 9 – ADAPTATION TO CELLVENTO BHK-200 SERUM FREE MEDIA: CONTROL CULTIVATION.
Growth (A) and metabolites (B) behavior of control BHK-21 cell line, in SCM. Cultivation was carried out during adaptation of BHK-21 cell line to Cellvento BHK-200 serum free media.

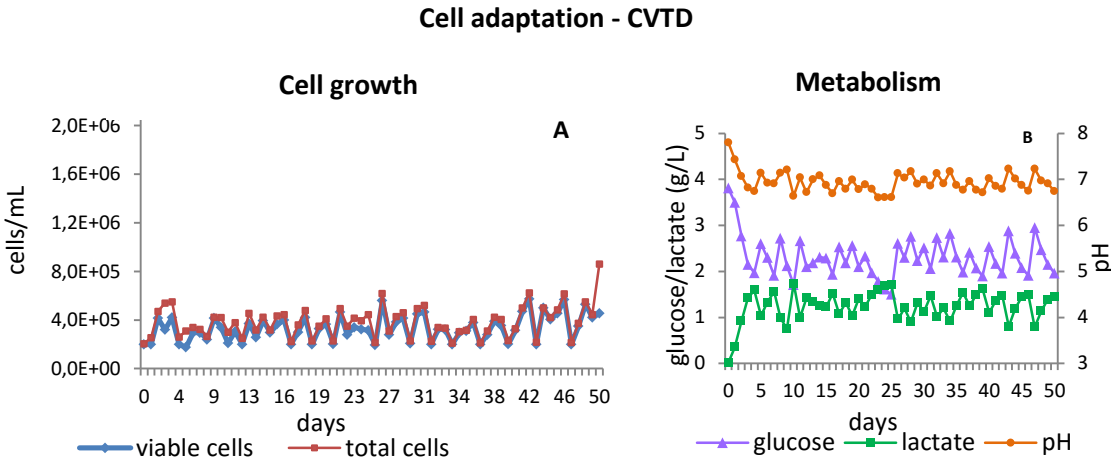

FIGURE 10 – ADAPTATION TO CELLVENTO BHK-200 SERUM FREE MEDIA BY DIRECT MEDIA CHANGE.
Growth (A) and metabolites (B) behavior of BHK-21 cell line, during adaptation to Cellvento BHK-200 serum free media, by direct media change.

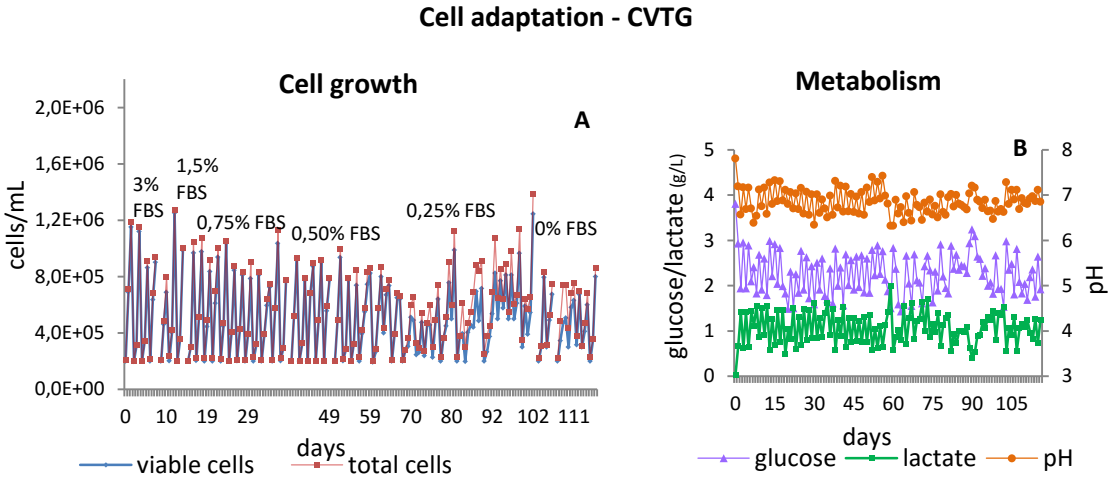

FIGURE 11 – ADAPTATION TO CELLVENTO BHK-200 SERUM FREE MEDIA BY GRADUAL SERUM REDUCTION.
Growth (A) and metabolites (B) behavior of BHK-21 cell line, during adaptation to Cellvento BHK-200 serum free media, by gradual serum reduction.

4.1.4. *Adaptation to* Cellvento BHK-200 from VP-SFM

- **Control culture**

Control culture presented standard behavior (Figures 12A and 12B) during subcultures. Cell morphology, size and refringence were normal. Cell density reached $7.5x10^5$ or higher after two days of cultivation, cell viability was higher than 80%, cells doubled or tripled every day. In some subcultures, cells quadrupled. Glucose consumption, lactate production and pH values were very similar during subcultures.

- **Direct Medium Change**

In adaptation using direct media change from VP to Cellvento BHK-200 (CVTDvp), first two passages presented much cells debris, but cell morphology, size and refringence remained normal. During subcultures, cells could not reach density higher than $7.5x10^5$, so in some subcultures innoculums with $5.0x10^5$ cells/mL were used, to make cells double and reach $1.0x10^6$ cells/mL (Figures 13A and 13B). In thirteenth subculture, cell density was $1.0x10^6$cells/mL, from innoculum of $2.0x10^5$cells/mL. Next two subcultures, cells maintained behavior, so, were submitted to sedimentation test. During sedimentation test, no clump was formed and culture reached density of $2.1x10^6$cells/mL.

After 42 days and 15 passages, cells were adapted to Cellvento BHK-200 culture medium and robust enough to virus production process.

- **Gradual Medium Change**

In adaptation using gradual media change from VP to Cellvento BHK-200 (CVTGvp), first step of media change was 75% of VP and 25% of Cellvento BHK-200, no alteration in cell growth behavior or metabolites was observed (Figures 14A and 14B), but in third passage cells grew in clumps. Even though, a new step of media substitution was done, 50% of VP and 50% of Cellvento BHK-200, no alteration in cell behavior or metabolites was observed. Next step was 25% of VP and 75% of Cellvento BHK-200. In this step, cells presented standard behavior. So next media change step was done, but, as adaptation to Cellvento failed in prior adaptation work, a more gradual media change was made in this adaptation process: 15% of VP and 85% of Cellvento BHK-200. In

this adaptation stage, no problem was observed. A new adaptation step was performed, where, 5% of VP and 95% of Cellvento BHK-200 were used. As, in this step, subculture had density of $1.1x10^6$cells/mL and viability of 99%, the last step was done, with 0% of VP and 100% of Cellvento BHK-200. Three subcultures were carried out, and no abnormal situation was detected. Cells were submitted to sedimentation test. During sedimentation test, no clump was formed and culture reached density of $1.9x10^6$ cells/mL. Cells were considered adapted to Cellvento BHK-200 culture media and robust enough to virus production. Seed bank was made.

Adaptation to Cellvento by gradual media change from cells adapted to VP took 42 days and 20 passages. CVTGvp adaptation took same time than CVTDvp adaptation, but more subcultures were done, this is due to in CVTDvp adaptation, cells took longer to reach cell density higher than $7.5x10^5$.

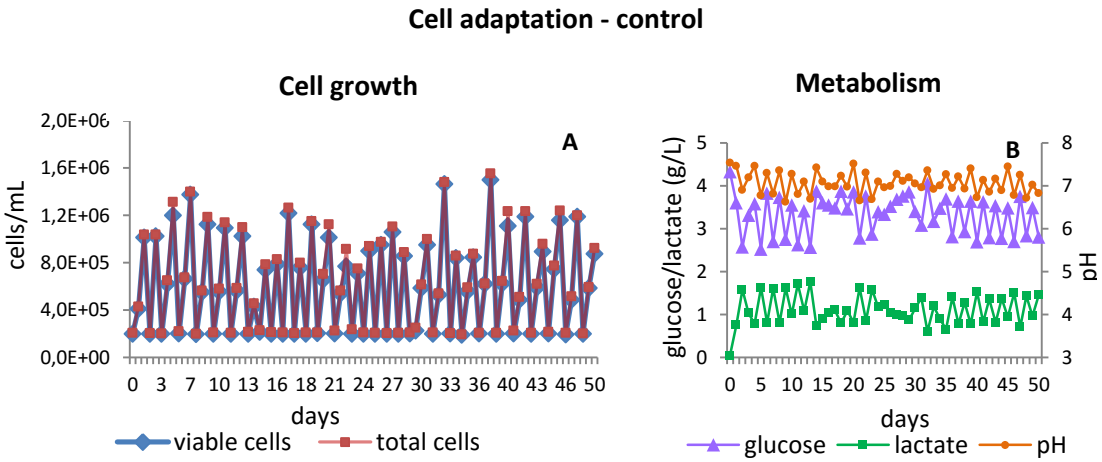

FIGURE 12 – ADAPTATION TO CELLVENTO BHK-200 SERUM FREE MEDIA, USING CELLS ADAPTED TO VP-SFM: CONTROL CULTIVATION.
Growth (A) and metabolites (B) behavior of control BHK-21 cell line, in SCM. Cultivation was carried out during adaptation of BHK-21 cell line to Cellvento BHK-200 serum free media, using cells previously adapted to VP-SFM.

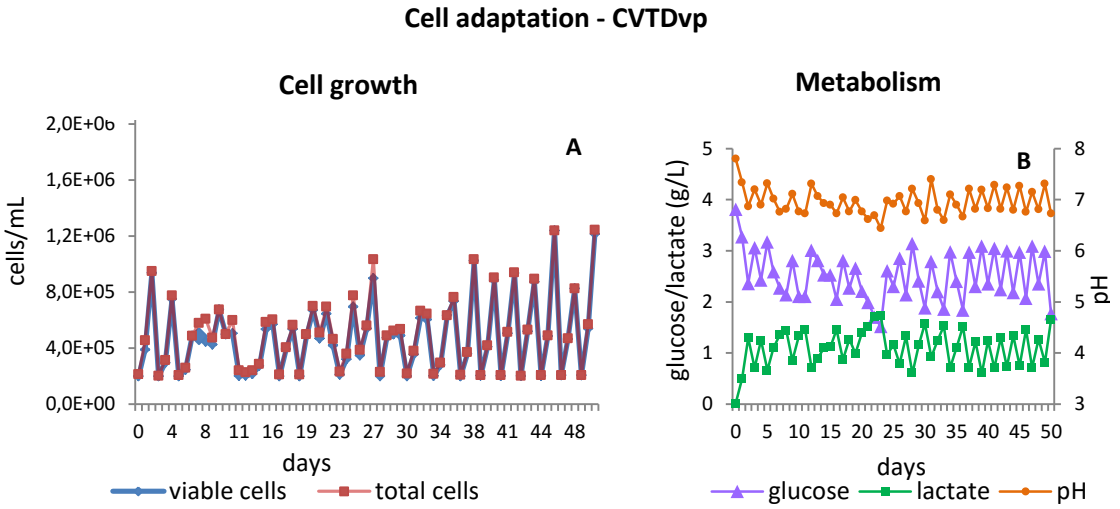

FIGURE 13 – ADAPTATION TO CELLVENTO BHK-200 SERUM FREE MEDIA BY DIRECT MEDIA CHANGE, USING CELLS ADAPTED TO VP-SFM.
Growth (A) and metabolites (B) behavior of BHK-21 cell line, during adaptation to Cellvento BHK-200 serum free media, by direct media change, using cells previously adapted to VP-SFM.

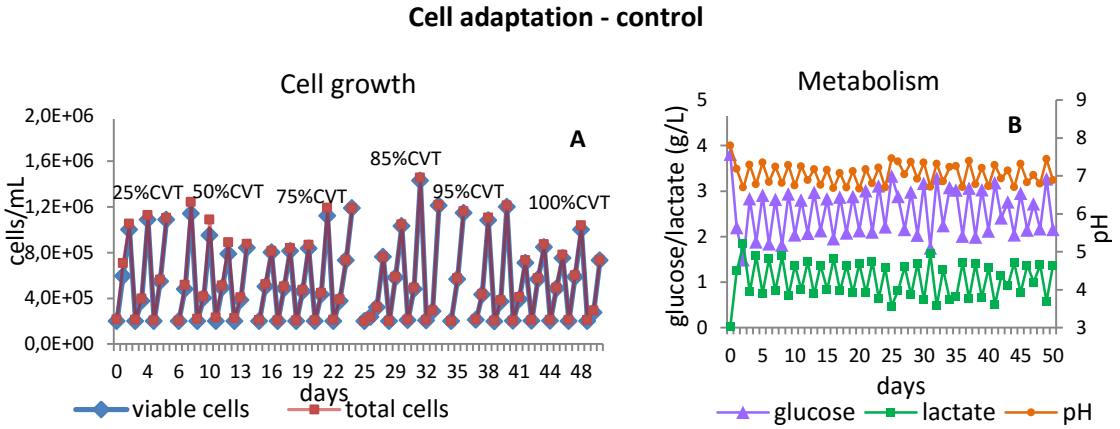

FIGURE 14 – ADAPTATION TO CELLVENTO BHK-200 SERUM FREE MEDIA BY GRADUAL MEDIA CHANGE, USING CELLS ADAPTED TO VP-SFM.
Growth (A) and metabolites (B) behavior of BHK-21 cell line, during adaptation to Cellvento BHK-200 serum free media, by gradual media change, using cells previously adapted to VP-SFM.

Table 3 presents a summary of cell adaptation to serum free media results. Considering that it was needed 26 days and 15 passages to adapt cells to VP-SFM, the actual value of passages to adapt BHK-21 cells to Cellvento BHK-200 culture media was 30 in direct media change, 35 in gradual media change, and it took 68 days.

TABLE 3 – SUMMARY OF CELL ADAPTATION TO SERUM FREE MEDIA.

	Media exchange			
Adaptation strategy	SCM→VP	SCM→Ex Cell	SCM→Cellvento	VP→Cellvento
Direct media change (D)	26 days 15 passages	36 days 14 passages	not adapted	42 days 15 passages
Gradual media change (G)	not adapted	36 days 18 passages	-	42 days 20 passages
Gradual serum reduction (S)	-	-	not adapted	-

It was expected that in direct media change cells would adapt to new media without need of subcultures. This result was not got in any culture media used. At least 14 passages for Ex Cell 302 and 15 passages for VP were needed to have cells adapted.

H. Kallel et al[19] adapted BHK-21 cell line to three commercial serum free media, HyQ PF CHO, HyQ PF CHO MPS and Ex Cell 302 in a single step by direct switch from serum containing media, and adapted BHK-21 to Rencyte by gradual media change. In gradual adaptation 26 days were necessary for adaptation. In this study, cells grew in monolayer. After adaptation, cells were no longer adherent, but grew in suspension forming clumps.

O. Merten et al[18] adapted BHK-21 cells to serum free media MD SS2, using same protocol that Kallel and collaborators[19] and cells also grew as clumps cultures.

In both studies, clumps cultures were able to produce rabies virus, however, in aggregated culture, it is not possible to have homogeneity, cells in the middle of aggregates have difficulties to access nutrients, and it is more difficult to have virus infection. Besides, cells in clumps do not grow as healthy as cells free in suspension. So cells in suspension adapted to serum free medium without forming clumps are a great perspective to industrial use.

4.2. Growth kinetics

Growth kinetics was carried out with all serum free adapted cell lines and with control culture, growing in SCM.

As shown in Figure 15, BHK-21 cells growth in SCM reached maximal viable cell density of $8.1x10^5$cells/mL in fourth day of cultivation. Glucose concentration was not limiting to growth nor lactate concentration reached inhibitory levels, following what was described by Cruz et al[25]. Cultivation lasted 6 days, where, there was no lag phase, 2 days of exponential phase, 3 days of stationary phase and 1 day of death phase. Maximal specific growth rate obtained was $0.036h^{-1}$. As glucose and lactate were not limitation to growth, probably cells stopped to grow due to ammonia production or by absence of any other nutrient, such as glutamine.

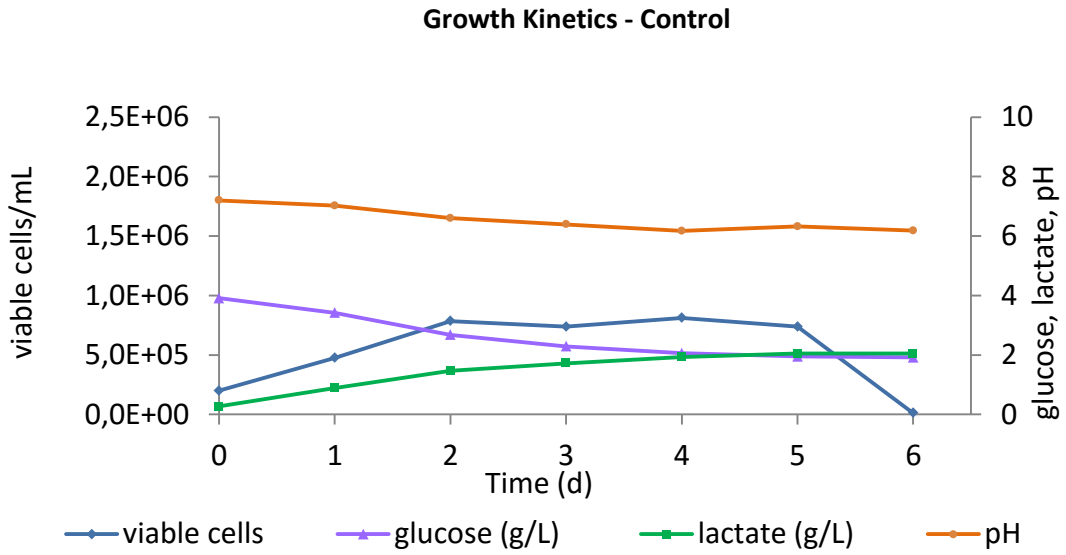

FIGURE 15 – GROWTH KINETICS OF BHK-21 CELLS IN SCM.

Cells adapted to VP-SFM, by direct media change (VPD) presented 1 day of lag phase, 1 day of log phase, 1 day of stationary phase and 2 days of death phase (Figure 16). Maximal cell density, $8.2x10^5$viable cells/mL, was obtained on third day of cultivation. Cultivation lasted 6 days, just like control culture. Growth was not limited by glucose nor lactate concentration25. As log phase was more acute, maximal specific growth rate obtained 0.052h-1 was higher than control culture. Cells lasted less time in stationary phase, and cell death was more gradual than in control culture. Maximal cell density was the lowest among serum free adapted cells (Table 4).

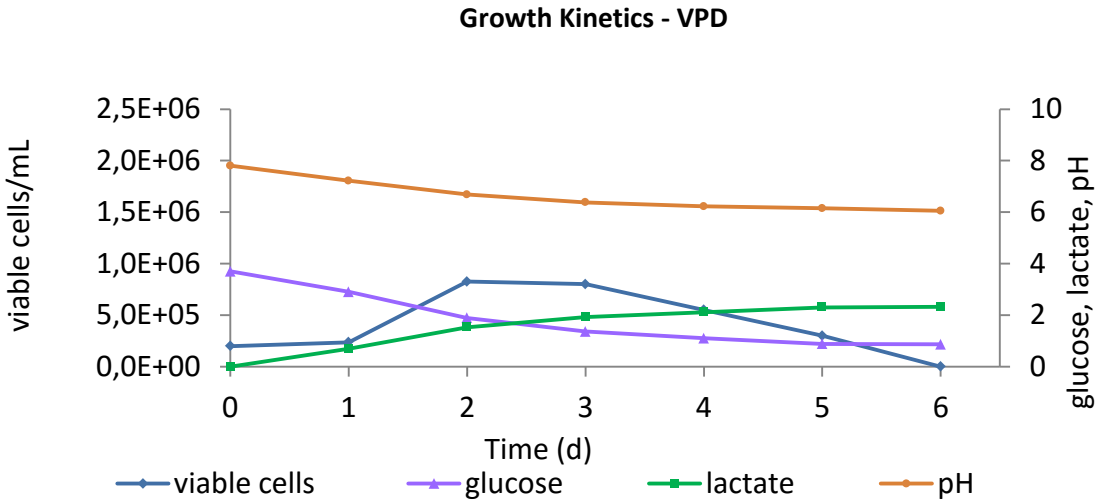

FIGURE 16 – GROWTH KINETICS OF BHK-21 CELLS ADAPTED TO VP-SFM BY DIRECT MEDIA CHANGE.

TABLE 4 – GROWTH OF BHK-21 CELLS IN SPINNER IN DIFFERENT SERUM FREE MEDIA AND DIFFERENT ADAPTATION STRATEGIES

	Maximal cell density (10^6 mL^{-1})	Maximal specific growth rate (h^{-1})	Time of cultivation (d)
Control	0,81	0,036	6
VPD	0,82	0,052	6
ExD	2,09	0,113	13
ExG	0,95	0,030	17
CVTDvp	1,03	0,058	8
CVTGvp	1,09	0,041	8

Cells adapted to Ex Cell 302 serum free media, by direct media change (ExD) presented an atypical behavior. In first day of cultivation, cell density was 1.95x106viable cells/mL, about 10 times higher than innoculum (Figure 17). Maximal specific growth rate was 0.113h-1, twice higher than highest obtained in other kinetics (Table 4). Maximal cell density, 2.09x106, was obtained on third cultivation day. This cultivation was the one with best energetic efficiency, since with similar glucose concentration available, higher cell density and lower lactate production were obtained, when compared with other growth kinetics. Glucose scarcity was obtained on fifth day, but cultivation lasted for 13 days, suggesting that other energy source, probably glutamine, was used during stationary phase. Death phase lasted only one day. Kallel et al19 also adapted

BHK-21 cells to Ex Cell 302 serum free media, but maximal cell density obtained was 7x105cells/mL and maximal specific growth rate obtained was 0.016h-1. This result suggests that protocol used for adaptation can interfere in cell growth behavior.

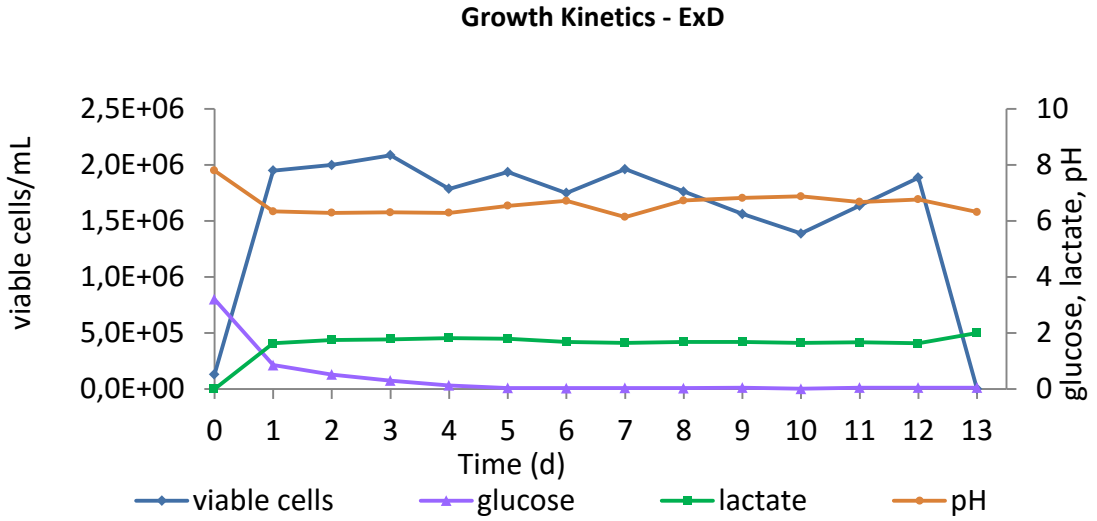

FIGURE 17 – GROWTH KINETICS OF BHK-21 CELLS ADAPTED TO EX CELL 302 BY DIRECT MEDIA CHANGE

Cells adapted to Ex Cell 302 serum free media, by gradual media change (ExG), reached maximal cell density, 9.5x105viable cells/mL, in thirteenth day (Figure 18). Cells growth was the slowest, with maximal specific growth of 0.030h-1, however, it had the longest cultivation time, lasting 17 days. This cultivation had 1 day of lag phase, 2 days of exponential phase, 12 days of maintenance phase, and 2 days of senescence phase. This cultivation reached the lowest pH value at the death phase, 6.01.

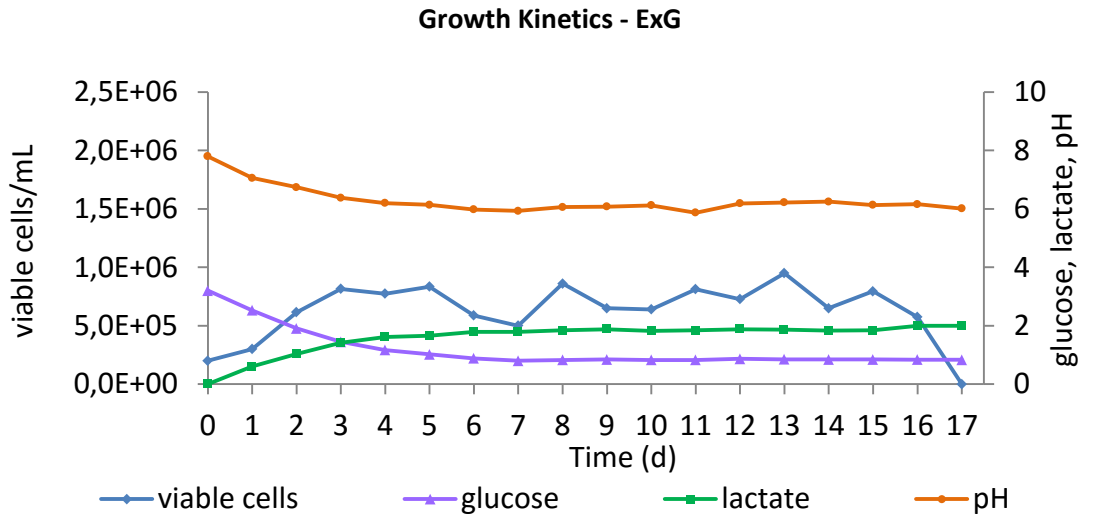

FIGURE 18 – GROWTH KINETICS OF BHK-21 CELLS ADAPTED TO EX CELL 302 BY GRADUAL MEDIA CHANGE.

Cells adapted to Cellvento BHK-200 media, by direct media change (CVTDvp) presented 1 day of lag phase, 3 days of log phase, no day in stationary phase and 4 days of death phase (Figure 19). Maximal cell density, 1.03x106viable cells/mL, was obtained on fourth day of cultivation. Cultivation lasted 8 days, and growth was neither limited by glucose nor lactate concentration19. Maximal specific growth rate obtained was 0.058h-1.

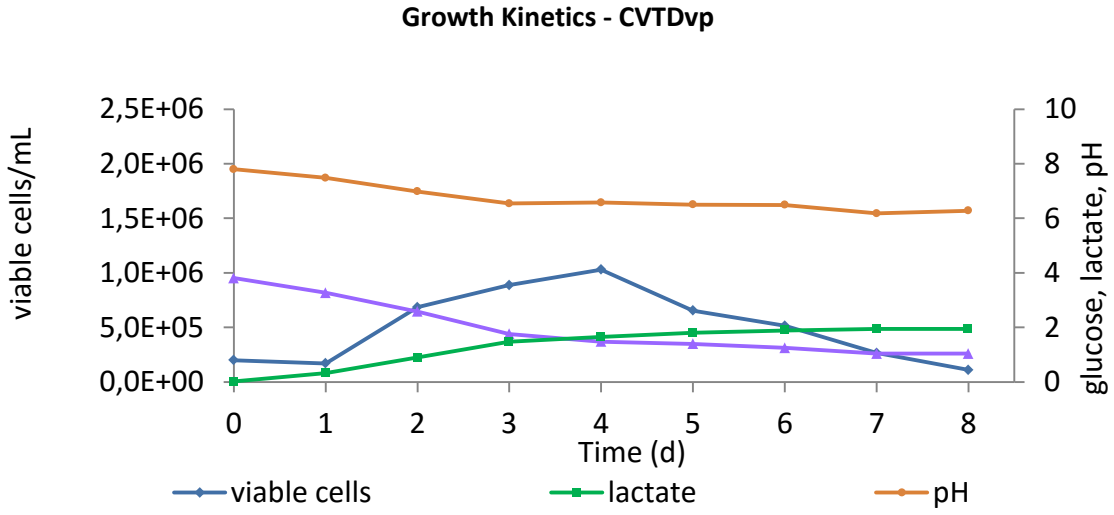

FIGURE 19 – GROWTH KINETICS OF BHK-21 CELLS ADAPTED TO CELLVENTO BHK-200 BY DIRECT MEDIA CHANGE, USING CELL PREVIOUSLY ADAPTED TO VP-SFM.

Cells adapted to Cellvento BHK-200 serum free media, by gradual media change (CVTGvp) presented very similar behavior to CVTDvp. One day of lag phase, 2 days of log phase, no day in stationary phase and 5 days of death phase (Figure 20). Maximal cell density, $1.09x10^6$viable cells/mL, was obtained on third day of cultivation. Cultivation lasted 8 days, and growth was neither limited by glucose nor by lactate concentration. Maximal specific growth rate obtained was $0.041h^{-1}$.

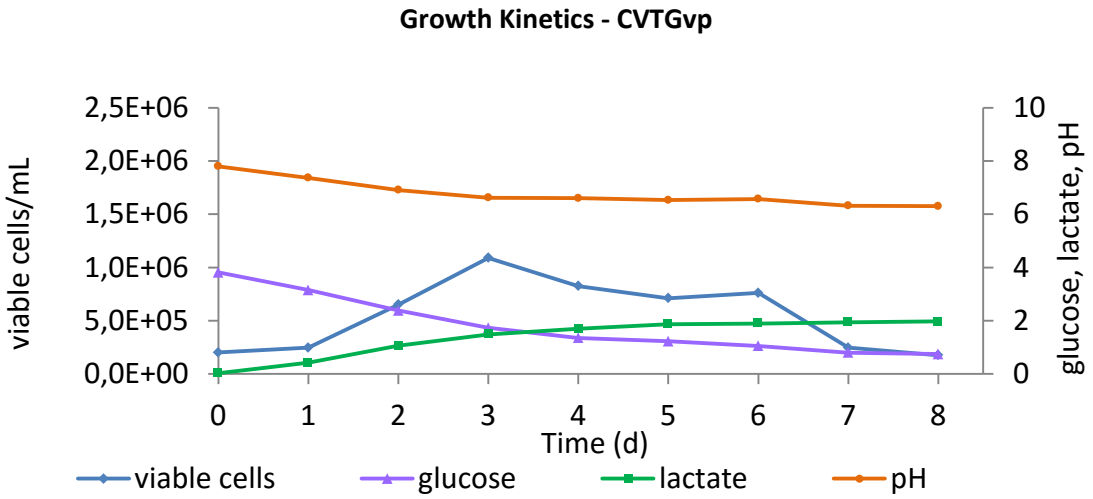

FIGURE 20 – GROWTH KINETICS OF BHK-21 CELLS ADAPTED TO CELLVENTO BHK-200 BY GRADUAL MEDIA CHANGE, USING CELL PREVIOUSLY ADAPTED TO VP-SFM.

Figure 21 shows a comparison among 6 kinetics studies. ExD cells cultivation obtained twice higher cell density and cultivation lasted twice longer than control cultivation. ExG cells cultivation lasted three times more than control culture with similar maximal cell density. Even though CVTDvp and CVTGvp did not have stationary phase, cells density were higher than control on 3rd to 6th cultivation days. VPD had maximal cell density similar to control culture, but cells deaths started earlier than control.

In this study, it is possible to affirm that BHK-21 cells are better adapted to Ex Cell 302 serum free culture media, and direct media change adaptation lead to a best cell adaptation.

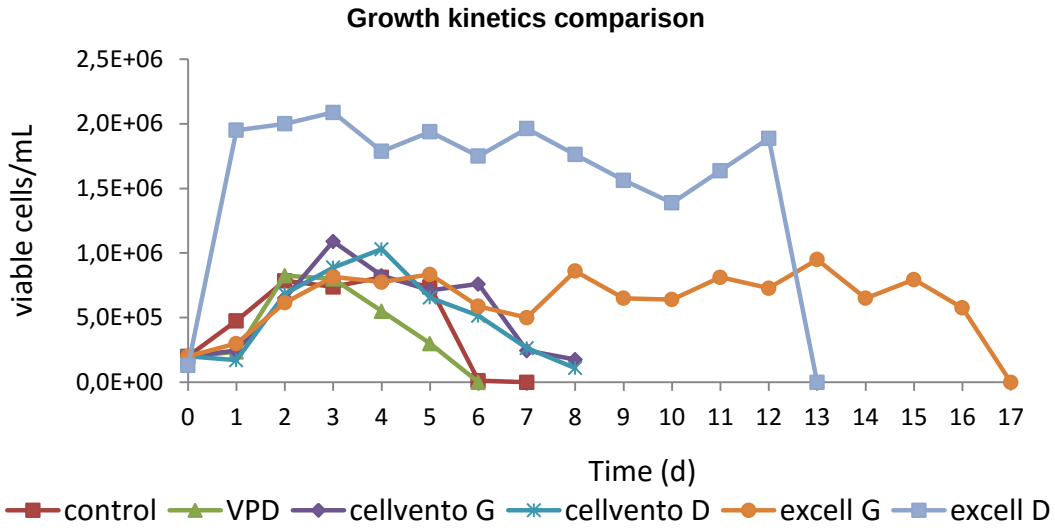

FIGURE 21- GROWTH KINETICS COMPARISON OF ALL ADAPTED CELL LINES.

4.3. Infection Kinetics

All adapted cell lines were submitted to infection kinetics. Control culture was carried out using SCMI.

During all infection kinetics studies, maximal cell densities of cultivations were obtained after infection, due to media harvest that provided nutrient and removed metabolites. In control cultivation, Figure 22A, cell viability decreased right after cell infection (total cells density became higher than viable cells density). Glucose and lactate metabolism after infection was very similar to metabolism of non infected cells (Figure 15). Since second day post infection (dpi), more than 75% of cells were infected. Titer peak (bold in Table 5) coincided with maximal cell density (Figure 22B), in third dpi.

Taking into account that in Tecpar, only harvest with $\log_{10}$ FFD_{50} higher than 3.8 are used to veterinary vaccine production, first harvest was the only one that does not meet this requirement (Table 5).

TABLE 5 – VIRUS TITER DURING INFECTION KINETICS OF ADAPTED CELLS TO SERUM FREE MEDIA.

Serum free adapted cells	Rabies virus titer $\log_{10}$ FFD_{50}/mL						
	1dpi	2dpi	3dpi	4dpi	5dpi	6dpi	average
Control	3,23	4,93	**5,47**	5,04	5,04	4,63	4,72
VPD	4,90	5,25	5,54	5,54	**5,62**	5,11	5,33
ExD	2,96	4,92	**5,54**	5,04	5,4	5,25	4,85
ExG	2,83	4,91	4,9	**5,32**	4,9	5,18	4,67
CVTDvp	3,53	4,63	4,9	**5,32**	**5,32**	4,64	4,72
CVTGvp	3,23	4,63	4,9	**5,05**	4,77	4,9	4,58

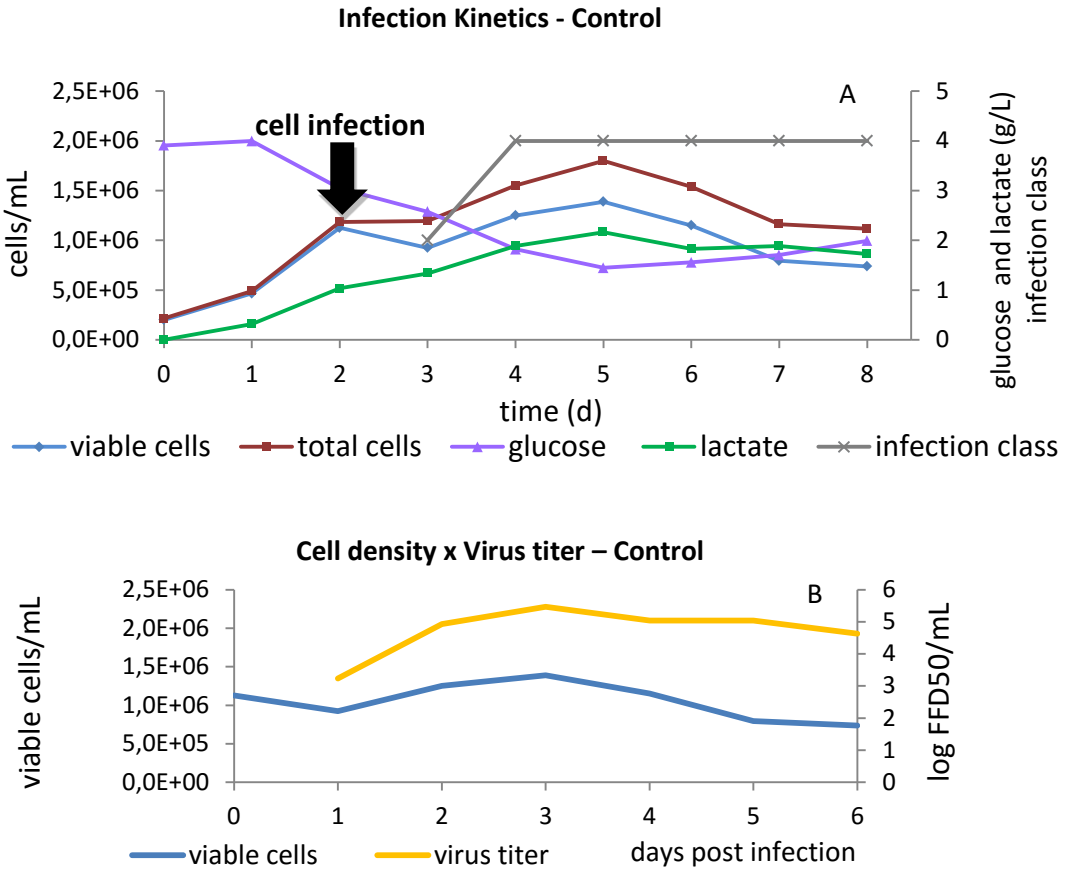

FIGURE 22 – INFECTION BEHAVIOR, CELL GROWTH AND METABOLISM AFTER RABIES VIRUS INFECTION (A), AND VIRUS PRODUCTION (B) OF CONTROL CELLS.

In Infection kinetics of VPD cells (Figure 23A), maximal cell density was $2.3x10^6$viable cells/mL. Cell viability decreased only after third dpi. Cells infection class IV was obtained on third dpi. After infection, glucose consumption increased when cells reached class IV of infection, all available glucose had been consumed, during harvests, media was replaced, and glucose was supplied, even though all glucose was consumed. This result may be explained because rabies virus is a glycoprotein, and glucose was used to produce it. Since first dpi, virus titer met vaccine production requirement. In this study, VPD infection reached highest virus titer and highest average of virus titer (Table 5). This cultivation was the only where maximal cell density has not coincided to virus titer peak (Figure 23B).

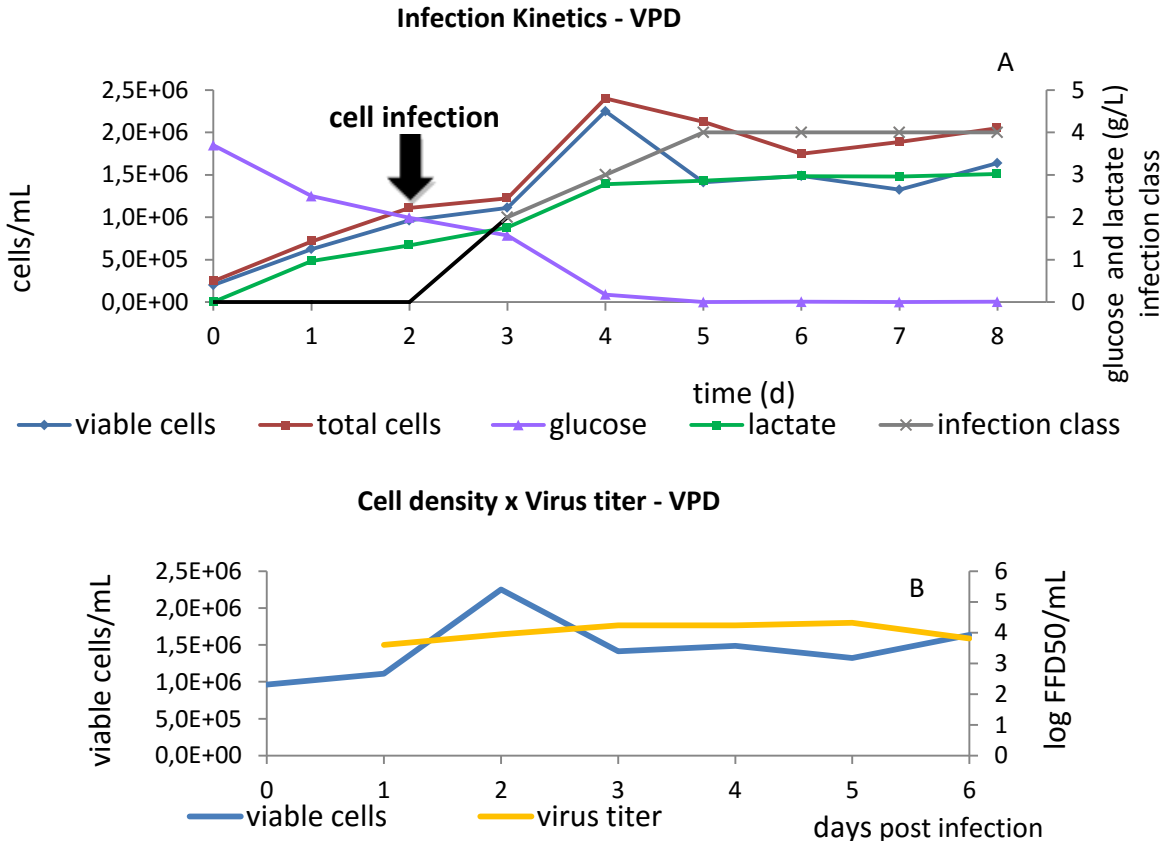

FIGURE 23 – INFECTION BEHAVIOR, CELL GROWTH AND METABOLISM AFTER RABIES VIRUS INFECTION (A), AND VIRUS PRODUCTION (B) OF CELLS ADAPTED TO VP-SFM BY DIRECT MEDIA CHANGE.

ExD infection presented maximal cell density 3 dpi (Figure 24A). A slight decrease of cell viability was observed right after cell infection. Virus titer peak (bold in Table 5) coincided with maximal cell density (Figure 24B) and when more than 75% of cells were infected. Glucose consumption and lactate production increased after infection, but glucose scarcity was not observed. Only first harvest has not reached minimal virus titer for vaccine production.

ExG infection reached maximal cell density on fourth dpi (Figure 25A). A slight decrease of cell viability was observed right after cell infection. Infection class IV was obtained on second day after infection. Virus titer peak (Table 5) coincided with maximal cell density (Figure 25B). Glucose consumption and lactate production increased after infection, but glucose scarcity was observed only on the sixth dpi. Only first harvest has not reached minimal virus titer for vaccine production.

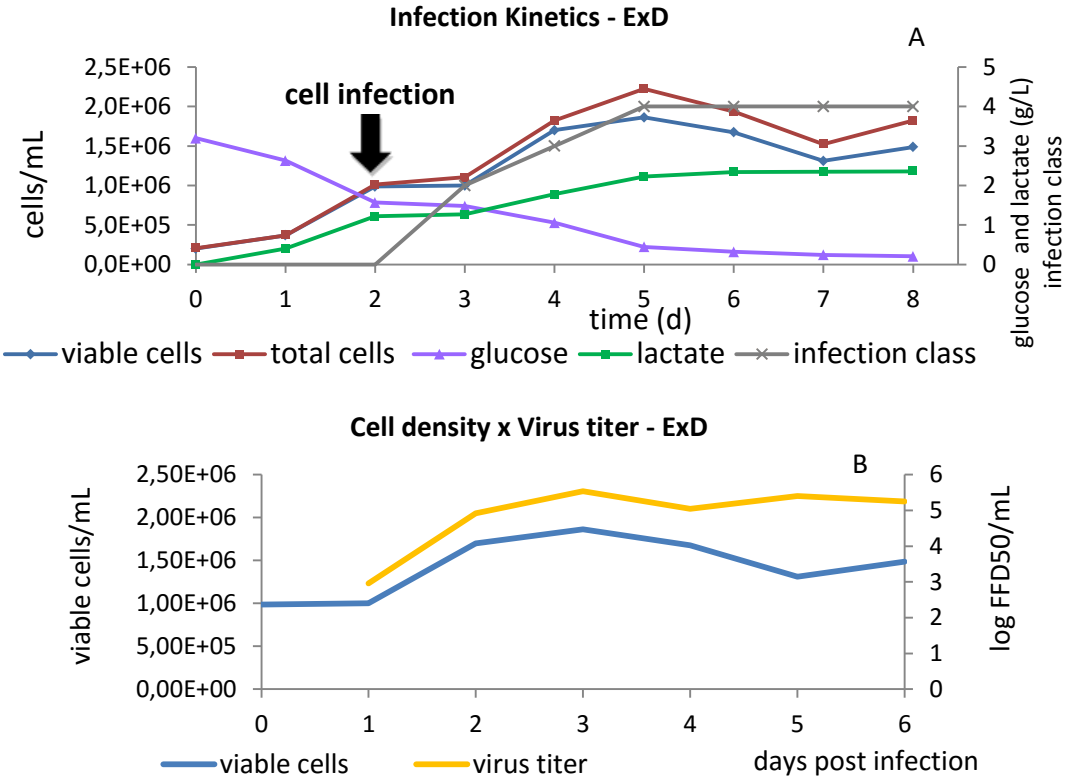

FIGURE 24 - INFECTION BEHAVIOR, CELL GROWTH AND METABOLISM AFTER RABIES VIRUS INFECTION (A), AND VIRUS PRODUCTION (B) OF CELLS ADAPTED TO EX CELL 302 BY DIRECT MEDIA CHANGE.

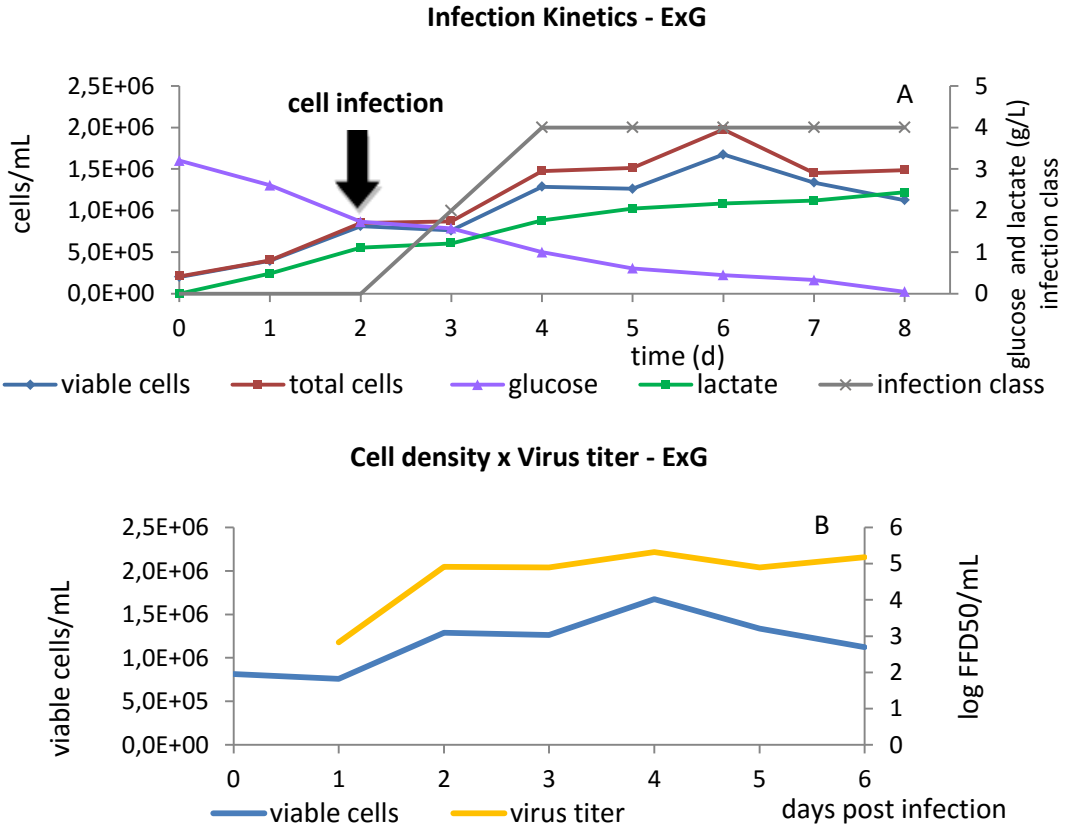

FIGURE 25 - INFECTION BEHAVIOR, CELL GROWTH AND METABOLISM AFTER RABIES VIRUS INFECTION (A), AND VIRUS PRODUCTION (B) OF CELLS ADAPTED TO EX CELL 302 BY GRADUAL MEDIA CHANGE.

CVTDvp infection presented maximal cell density 5 days post infection (Figure 26A). A high decrease of cell viability was observed right after cell infection. Virus titer peak (bold in Table 5) coincided with maximal total cell density, at fourth dpi, and with maximal viable cell density, on 5 dpi (Figure 26B). One day after infection, less than 25% of cells were infected. Only on third dpi more than 75% of cells were infected. Glucose consumption and lactate production increased after infection. Glucose scarcity was only observed at sixth harvest. First harvest was the only that did not reached minimal virus titer for vaccine production.

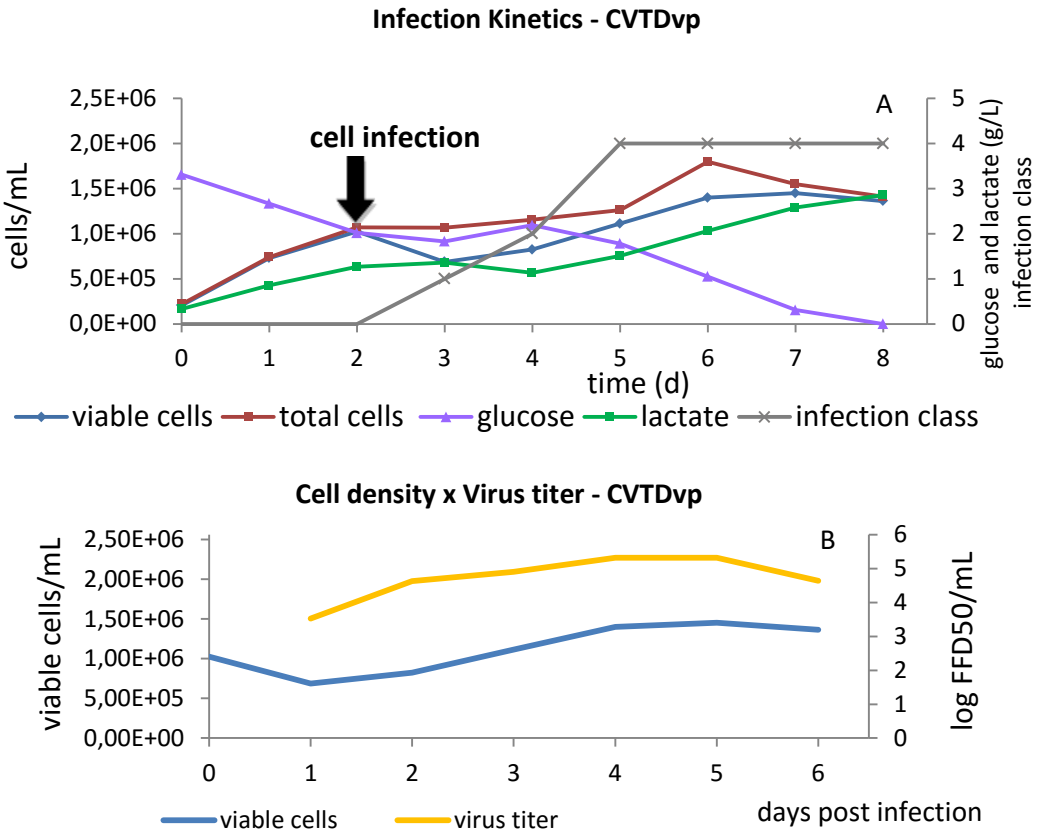

FIGURE 26 - INFECTION BEHAVIOR, CELL GROWTH AND METABOLISM AFTER RABIES VIRUS INFECTION (A), AND VIRUS PRODUCTION (B) OF CELLS ADAPTED TO CELLVENTO BHK-200 BY DIRECT MEDIA CHANGE.

CVTGvp infection presented maximal cell density 4 days post infection (Figure 27A), coinciding with virus titer peak (Figure 27B). A high decrease of cell viability was observed right after cell infection. One day after infection, less than 25% of cells were infected. More than 75% of cells infection was observed

on third dpi. Glucose consumption and lactate production increased after infection, but glucose scarcity was only observed at sixth harvest. Only first harvest has not reached minimal virus titer for vaccine production.

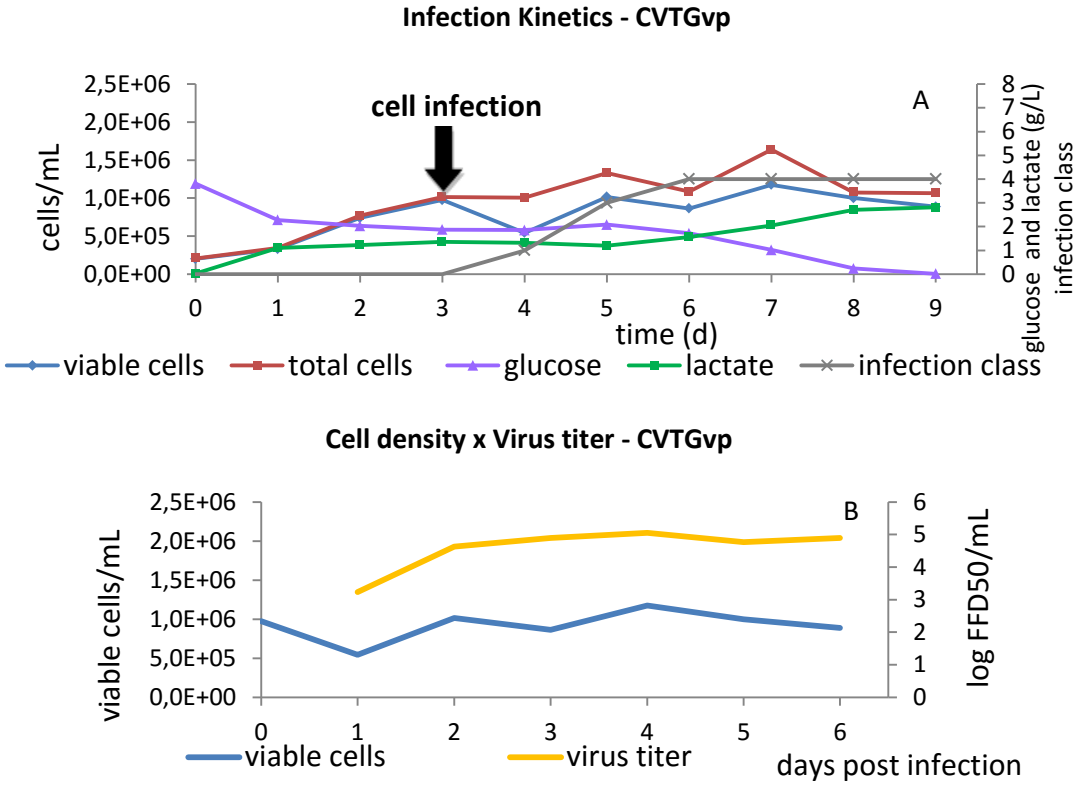

FIGURE 27- INFECTION BEHAVIOR, CELL GROWTH AND METABOLISM AFTER RABIES VIRUS INFECTION (A), AND VIRUS PRODUCTION (B) OF CELLS ADAPTED TO CELLVENTO BHK-200 BY GRADUAL MEDIA CHANGE.

Figure 28 shows virus titer during infection kinetics of 6 studies. With exception of VPD, that had all harvests with virus titer meeting requirements for vaccine production, others presented enough virus titer since second dpi.

No relation was observed between infection class and virus titer, since, even when less than 50% of cells were infected, virus titer was higher than $10^{3.8}$. All adapted cells showed capacity to produce virus for vaccine production.

Cell adapted to VP culture media, presented worst growth kinetic, but best virus production, demonstrating the importance of virus infection study. VP is a culture media developed to virus production, and probably presents formulation more appropriated for this indication. Studies should be carried out to

investigate if increasing glucose supply during infection would enhance virus production.

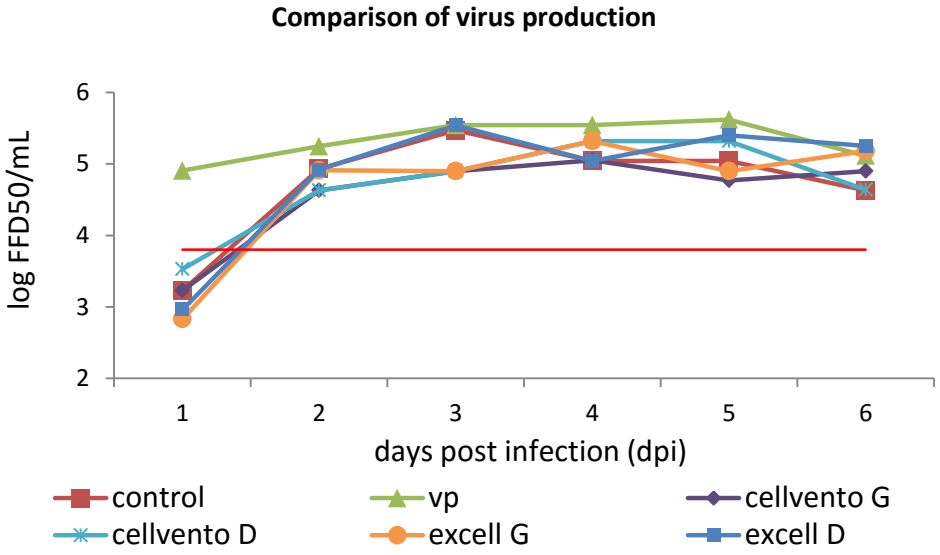

FIGURE 28 - COMPARISON OF VIRUS PRODUCTION OF ALL ADAPTED CELL LINES. Red line is cut off line. Only results above read line are used for vaccine production

Results presented indicate that adapted to serum free cells can produce rabies virus for vaccine production. However, virus harvests have to formulated and challenged in animals to confirm vaccine potency.

Perrin et al[17] carried out cell infection in bioreactor using perfusion system. Best virus titer were achieved from third to fifth days after infection. Results of this study have presented high virus titer from second until sixth day post infection.

Kalel et al[19] infected BHK-21 adapted to serum free media with rabies virus. Study was carried out in spinners. Virus titer started to increase after three and five days post infection, depending on media used. Virus peaks were obtained on ninth dpi.

Although cells adapted to VP-SFM culture media showed best virus titers results, attention should be paid to Cellvento BHK-200 culture media. As this culture media was developed to veterinary vaccine, mainly mouth-and-foot decease, that requires large scale production and very low prices, when compared to human vaccines, Cellvento BHK-200 price is about ten times cheaper than other serum free media that were developed for human viral

vaccine production (VP-SFM), or for recombinant proteins for human use (Ex Cell 302).

Table 6 shows a comparison of culture media costs using serum free media to produce 10 millions of doses of rabies vaccine. So, until this moment, Cellvento BHK-200 is culture media seems to be the most adequate for veterinary rabies vaccine production.

TABLE 6 – COST ESTIMATION OF SERUM FREE CULTURE MEDIA FOR 10 MILLION OF DOSES OF VACCINE PRODUCTION.

Culture medium	**Volume for 10 million of doses**	**Medium costs (EUR)**
VP-SFM	10,000 L	400,000.00
Ex Cell 302	11,000L	440,000.00
Cellvento BHK-200	11,000L	**40,000.00**

5. Conclusion

BHK-21 C13 cell in suspension was able to adapt in the three serum free culture media used in this study (VP-SFM, Ex-Cell 302 and Cellvento BHK-200). Kinetics studies of cell growth and rabies virus production demonstrated that best results in growth kinetics does not mean best results in virus production. All adapted cells are able to produce rabies virus for vaccine production. Different adaptation protocols do not interfere on results of virus production. Best virus productivity was reached using VP-SFM culture media, however, taking into account economic aspects, best result achieved was by using Cellvento BHK-200.

Vaccine potency must be determined to prove virus quality. Since vaccine potency is good, scale up studies using serum free media should be carried out to permit serum substitution in industrial veterinary rabies vaccine production.

BIBLIOGRAPHIC REFERENCES

1 WHO **Expert Consultation on Rabies: second report**. WHO technical report series no. 982, Geneva, World Health Organization, 2013.

2 Hemachudha T., Laothamatas J., Rupprecht C.E. **Human rabies: a disease of complex neuropathogenetic mechanisms and diagnostic challenges**. Lancet Neurology, 2002, 1(2):101–109.

3 Ugolini G. **Rabies virus as a transneuronal tracer of neuronal connections**. Advances in Virus Research, 2011, 79:165–202.

4 WHO **Expert Consultation on Rabies: first report**. WHO technical report series no. 931, Geneva, World Health Organization, 2004.

5 King A.A. and Turner G.S. **Rabies a review**. Journal of Comparative Pathology, 1993,108:1-39.

6 Fuenzalida, E. and Palacios, R. **Un método mejorado para la preparación de la vacuna antirabica**. Bulletin of the Institute of Bacteriology, Chile, 1955, 8:3.

7 Mello, I.M.V.G.C., Conceição, M.M., Jorge, S.A.C., Cruz, P.E., Alves, P.M.M., Carrondo M.J.T., and Pereira C.A. **Viral vaccines: concepts, principles, and bioprocesses**. In Castilho, L.R., Moraes, A.M., Augusto, E.F.P. and Butler, M. (Ed.) Animal cell technology: from biopharmaceuticals to gene therapy. New York: Taylor & Francis Group, 2008, 435-458.

8 WHO **Rabies vaccines: WHO position paper**. Weekly epidemiological record n. 32, Geneva, World Health Organization, 2010.

9 Diamond, L. **Two spontaneously transformed cell lines derived from the same hamster embryo culture**. *J.* Cancer, 1967, 2:143.

10 Precausta, P. **Vaccin antirabique inactivé á usage vétérinaire preparé à partir de culture cellulaire**. Symp. Series Immunobiol. Standard., 1974, 21:162.

11 Macpherson I. and Stoker M., **Polyoma transformation of hamster cell clones - an investigation of genetic factors affecting cell competence**. Virology 1962, 16:147-151.

12 Pay, T., Boge, A., Menard, F. and Radlett, P. **Production of rabies vaccine by an industrial scale BHK-21 suspension cell culture system**. Develom. Biol. Stand., 1985, 60:171.

13 Léo P., Galesi A.L.L., Suazo, C.A.T. and Moraes, A.M. **Animal cells: basic concepts**. In Castilho, L.R., Moraes, A.M., Augusto, E.F.P. and Butler, M.

(Ed.) Animal cell technology: from biopharmaceuticals to gene therapy. New York: Taylor & Francis Group, 2008, 13-38.

14 Moraes, A.M., Mendonça, R.Z. and Suazo, C.A.T. **Culture media for animal cells**. In Castilho, L.R., Moraes, A.M., Augusto, E.F.P. and Butler, M. (Ed.) Animal cell technology: from biopharmaceuticals to gene therapy. New York: Taylor & Francis Group, 2008, 111-128.

15 Ohmori, K., Masuda, K, Maeda,S., Kaburagi,Y., Kurata K., Ohno, K., DeBoer, D.J., Tsujimoto, H. and Sakaguchi M. **IgE reactivity to vaccine components in dogs that developed immediate-type allergic reactions after vaccination**. Veterinary Immunology and Immunopathology 2005, 104:249–256

16 Ohmori, K., Masuda, K, Maeda,S., Ohno, K., DeBoer, D.J., Sakaguchi M. and Tsujimoto, H. **Immunoblot analysis for IgE-reactive components of fetal calf serum in dogs that developed allergic reactions after non-rabies vaccination**. Veterinary Immunology and Immunopathology 2007, 115:166–171.

17 Perrin, P., Madhusudana, S., Gontier-Jallet C., Petres, S., Tordo, N and Merten, O. **An experimental rabies vaccine produced with a new BHK-21 suspension cell culture process: use of serum-free medium and perfusion-reactor system**. Vaccine, 1995, 13:1244-1250.

18 Merten, O.-W., Kierulff, J., Castignolles, N. and Perrin, P. **Evaluation of the new serum-free medium (MDSSP) for the production of different biologicals: use of various cell lines**. Cytotechnology, 1994, 14:47.

19 Kallel, H., Jouini, A., Majoul, S, Rourou S. **Evaluation of various serum and animal protein free media for the production of a veterinary rabies vaccine in BHK-21 cells**. Journal of Biotechnology 2002, 95:195–204.

20 Batista, A. M., Cruz, P. S., Almeida, E., Costa, A. E. B., Scheffer, K. C., Chaves, L. B., Silva, A. C. R., Caporale, G. M. M. **Infection of BHK-21 cells cultivated in stationary monolayers by PV and CVS strains**. Boletin Epidemiologico Paulista (BEPA), 2009, 6:71.

21 Smith, J. S. Yager, P.A., Baer, G.M., **A rapid fluorescent focus inhibition test (RFFIT) for determine rabies virus-neutralizing antibody**. In: Meslin, F. X., Kaplan, M.M., Koproeski, H (Eds.), Laboratory Techniques in Rabies, 4th ed. Geneva, World Health Organization, 1996, 181-192.

22 Moura W. C., Gallina N. M. F. G., Fuches R. M. M., Romijn P. C., Leite J. P. G. **Validation of a virus neutralization potency test in BHK-21 cells for rabies immunoglobulins in a two-center study**. Journal of Virological Methods, 2008, 154:7-13.

23 Fuches, R.M.M. **Validação de bioensaios para o estudo da imunogenicidade da vacina contra raiva**. Tese (Doutorado na área de Biotecnologia) – Universidade de São Paulo/Instituto Butantan/Instituto de Pesquisas Tecnologícas., 2010.

24 Lorenz, R.J., Bogël, K., **Methods of calculation**. In Meslin, F. X., Kaplan, M.M., Koproeski, H (Eds.), Laboratory Techniques in Rabies, 4th ed. Geneva, World Health Organization, 1996, 321-335.

25 Cruz, H.J., Freitas, C.M., Alves, P.M., Moreira, J.L., Carrondo, M.J.T. **Effects of ammonia and lactate on growth, metabolism and productivity of BHK cells**. Enzyme Microb. Technol. 2000, 27:43–52.

Internet references:

26 Hunt, R., **Virology – Chapter twenty – Rabies**. Micobiology and Immunology Online, University of South Carolina School of Medicine, 2011. Available at:

< http://pathmicro.med.sc.edu/virol/rabies.htm> Accessed on: September, 2013.

27 Merck Millipore, Product Information: 1.02376 Cellvento™ BHK-200 cell culture medium. Available at:

<http://www.merckmillipore.com/showBrochure?id=201307.058> Accessed on: September, 2012.

28 SAFC Bioscience, **Product information Ex-Cell 302 serum-free medium for CHO cells**. Availabel at:

<http://www.sigmaaldrich.com/etc/medialib/docs/Sigma/Product_Information_Sheet/p14326.Par.0001.File.tmp/p14326.pdf> Accessed on: September, 2012.

29 Gibco, **VP-SFM**, July, 2008. Available at:

<http://tools.lifetechnologies.com/content/sfs/manuals/3918%20VP%20SFM.pdf>

Accessed on: September, 2012.