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Application of a Serum/Protein-Free Medium for the Growth of Mammalian Cell Lines and High Level Production of Classical, Variant and Very Virulent Strain of Infectious Bursal Disease Virus (IBDV)

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Abstract: The aim of this study was to verify the capacity of IBDV strains, Lukert, F52/70 and very virulent Brazilian genotype (G11) to infect and replicate on cell culture maintained without serum or protein supplementation. BHK_{21} and CER cells were directed adapted to growth under absence of protein and/or fetal bovine serum with addition of 5 μg mL^{-1} of insulin-like growth factor I (IGF-I). The RPMI 1640 medium plus IGF-I was able to support both cell lines growth at a maximum of $3{,}3\times10^6$ and $3{,}4\times10^6$ cells mL^{-1} for CER and BHK_{21}, respectively. The comparison between RPMI 1640 medium plus IGF-I and conventional MEM plus fetal bovine serum (FBS) growth curves demonstrated no significant difference, however the lowest surveillance of BHK_{21} and CER cells under MEM plus BFS growth Both cells provided infectious virus titres of up to $10^{5.5}$ 50% tissue culture infective doses 100 μL^{-1} ($TCID_{50}$ 100 μL^{-1}). Furthermore, they prove to be susceptible to infection with three different IBDV strains by culture both cells with RPMI 1640 medium plus IGF-I medium. It was concluded that both cells may be used for laboratory multiplication of IBDV strains without any serum and/or animal protein with a considerable ethical impact.

Key words: CER cells, IGF-I, serum-free medium, extra-cellular matrix proteins

INTRODUCTION

Infectious bursal disease virus (IBDV) is a member of the genus *Avibirnavirus* in the family of *Birnaviridae*. IBDV has two distinct serotypes, serotype 1 and serotype 2[1]. Serotypes 1 viruses are pathogenic for chickens, whereas serotype 2 viruses are avirulent. Numerous serotype 1 strains may cause Infectious Bursal Disease (IBD) in chickens and display a wide range of immunosuppressive effects and pathogenicity in chickens. Thus, serotype 1 strains can be classified as apathogenic, mild, intermediate, classic variants and very virulent[2]. Since the mid 1980's a new very virulent strain of IBDV (vvIBDV) has emerged, first in the Netherlands and after disseminated to other European countries and unfortunately as well as worldwide. In order to overcome this, more IBDV virulent strains have been introduced as vaccines in Brazil, increasing the risk of immunossupression, clinical disease and/or new serotypes in the field. In addition, high cost of these vaccination programs, vaccine development and heavy economic losses due to the failure of these vaccinations, leads the IBDV as the most important avian pathology worldwide[3]. Actually, attempts have been made to develop techniques for IBDV infection and new cell lines have been proposed for its isolation, replication and vaccine production[3,4]. Moreover, the Lukert strain has been adapted to replicate and produce cytophatic effects in primary cell cultures, including chicken bursa lymphoid cells, chicken embryo kidney cells and chicken embryo fibroblast cells[3]. Usually, growth factors, individually, are used on a primary cell culture system to increase cell surveillance by replacing the Foetal Bovine Serum (FBS) supplementation[5,6]. The FBS has traditionally been used in cell culture, for a long time, as an essential nutrient, normally mixed to the culture medium (growth factors and hormones) which allow attachment and spread of the cells, produced by reverse genetic systems[7]. In addition, these components play an

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important role in maintain the microenvironment regulating cell behaviour *in vitro*[8,9]. The most widely used animal serum supplement is fetal bovine, characterized as an ill-defined component in cell culture media, which improved the development of chemically defined serum-free media formulations in the last two decades. Continuous cells line have been frequently used for *in vitro* assays, cultured on serum/protein media, considered as a standard protocol. In spite of the FBS advantages, the amount of this product for the world market is estimated at approximately 500.000 L $year^{-1}$. This means, that more than 1.000.000 bovine fetuses have to be harvested and it is expected, that these numbers will continue to increase annually[7]. Contemporary knowledge in modern cell biology and biotechnology allowed the identification of growth factors involved in cell proliferation, maturation and differentiation. One of these growth factors well sudied is insulin-like growth factor I (IGF-I), produced by *Escherichia coli* in a reverse genetic system and herein, was added to RPMI 1640 commercial medium to support BHK_{21} and CER growth infected by three IBDVs serotypes.

MATERIALS AND METHODS

Adaptation of BHK_{21} and CER cell lines to serum/protein free medium: The following CER cells (chicken embryo related cell line, hybrid derived from chicken embryo fibroblast and BHK_{21}) was used and gently supplied by Laboratório de Virologia, UNICAMP, São Paulo, Brazil. The BHK_{21} (Baby Hamster Kidney cell clone 21) was purchased from ATCC by Instituto Biológico São Paulo, Brazil. First, all cells were growth in flasks of 25 cm^3 at 37°C, using an initial concentration of 2.8×10^4 cells mL^{-1} in a traditional Minimal Essential Medium (MEM, GIBCO-BRL) plus antibiotics and 10% of FBS (GIBCO-BRL).The cells were switched to serum/free medium, RPMI 1640 (GIBCO-BRL) plus 5 μg mL^{-1} of insulin growth factor I (IGF-I) as described before[10]. After 5 consecutive passages, the cells were cultured in flasks of 25 cm^3 at 37°C, using an initial concentration of 2.8×10^3 cells mL^{-1} and samples were taken at time 0, 24, 48, 72, 96, 120, 144, 168, 192, 216 and 240 h post-seeding. The respective supernatant, of all cell culture were collected and harvest for *Mycoplasma* sp. detection. The chicken embryo fibroblast (CEF) culture was included as mock cells only for RNA quantification.

Growth characteristics in BHK21 and CER cell lines All cells, BHK_{21} and CER, growth as monolayer received 0.5 mL of a trypsin/versene solution and 0.5 mL of cell sample were taken. After incubation at 37°C for 10 min, cells were staining with tryplan blue (0.2% w/v in PBS) and counted using a hemacytometer and the results expressed as cells mL^{-1} (cells mL^{-1}). The samples were taken in triplicate at 0, 24, 48, 72, 96, 120, 144, 168, 192, 216 and 240 h post-seeding for RPMI 1640 plus IGF-I and MEM plus FBS.

Susceptibility of BHK21 and CER cells to IBDVs strains infection: Stocks of F52/70, Lukert and G11 (Brazilian genotype of vvIBDV) were produced by infecting 3 week old chickens. All IBDV strains used in this study were kindly supplied by Dr. Clovis Oliveira (Merial Laboratories). The Lukert, F52/70 and G11vv IBDV strains were used to infected healthy, static confluent monolayer of CER and BHK_{21} cells, using 0.5 mL of virus suspension for infection of 75 cm^3 flasks, previously filtrated by 0.22 μm of exclusion (Corning) with multiplicity of infection of 1, which had been previously determined for both cells. Adsorption was allowed for 1 h at 37°C, the inoculum was removed and IBDV strains were submitted to five consecutive passages for adaptation. The monolayers were then washed with fresh medium and incubated to different time intervals (0, 24, 48, 72, 96, 120, 144, 168, 192, 216 and 240 h post-infection, p.i.), incubated at 37°C in 5% CO_2 atmosphere in humidified incubator. The supernatants were harvested, freeze-thawed three times and centrifuged 2,000 g for 10 min and assayed for the presence of the virus. All experiments were performed in triplicates. Infectious virus titres were calculated according to Spermann-Kärber method and expressed as $\log_{10}$ $TCID_{50}/50\mu L$[11].

Real time quantitative RT-PCR: The RNA, reverse transcription reaction, primers and PCR conditions were followed by recommendations previously reported[12]. The List of primers, size and number access number of all sequences analyzed by real time RT-PCR SYBR green technology are described (Table 1). The real time quantitative PCR was performed in the iCycler iQ real-time PCR detection system (Bio-Rad, USA) using the SYBR green dye. All

Table 1: List of primers, size and number access number of all sequences analyzed by real time RT-PCR SYBR green technology

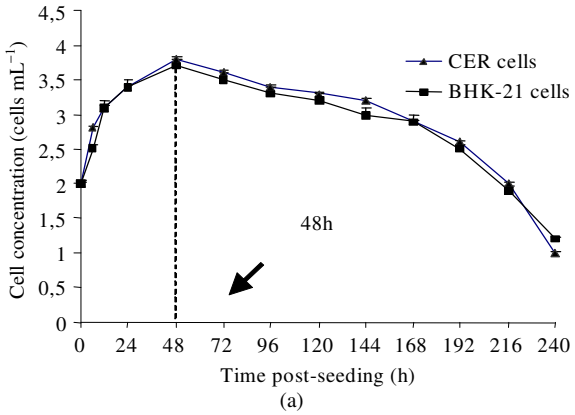
Gen	Primer	Sequences	Size
β-actin	FW	GAGAAATTGTGCGTGACATCA	152 pb
	RV	CCTGAACCTCTCATTGCCA	
F52/70	FW	AGATAACCCAGCCAATCAC	173 pb
	RV	CACTCTTTCGTAGGCTACTAGT	
Lukert	FW	AGATAACCCAGCCAATCAC	166 pb
	RV	TCGTAGGCCACTAGCGT	
G11	FW	GCCCAGATCTACACCAT	743 pb
	RV	CCCGGATTATGTCTTTGA	

standard dilutions, controls and infected samples were run in triplicate and average value of relative copy number was used to quantify IBDVs RNA. The chicken embryo fibroblast cells (CEF) derived from specific pathogen chicken embryos infected by all IBDVs strains, were used for the positive standard control. Total cellular RNA from supernatant of infected cells at 60h post-infection was isolated using the Trizol® method and the isolated RNA eluted in 20 μL of Rnase-free water (Invitrogen™). The RNA concentration was quantified using the spectrophotometer at 280nm and to eliminate any possible contamination 0.1 U μL^{-1} of Dnase (Invitrogen™) was applied. The first reverse transcription (RT) reaction was performed using Superscript SIII first strand Kit (Invitrogen™) according to the manufacture's instructions. The RT products were diluted 10 times and then stored at -20°C or further PCR. Sequences of classical Lukert, F52/70 and vvIBDV genome were used to design specific primers and number in GenBank and size of PCR are shown in Table 1. The beta (β) actin was applied as internal control of real time RT-PCR. The viral amount in the samples was evaluated by relative quantification to β-actin and column plot of viral amount was made by use Microsoft Excell® software[12].

Statistical analysis: Statistical analysis was performed using the Student´s t-test and analysis of variance (ANOVA); the least significant difference at p = 0.05 was determined. Statistical analysis was performed with Minitab® for Windows Release 11.1 (Minitab Inc., State College, PA, USA).

RESULTS AND DISCUSSION

Growth curves of CER and BHK_{21} cells: Mammalians cell lines (CER and BHK_{21}) were susceptible to growth by the use of RPMI 1640 plus IGF-I. The growth curve of both cell lines obtained after direct adapted to RPMI 1640 medium plus IGF-I



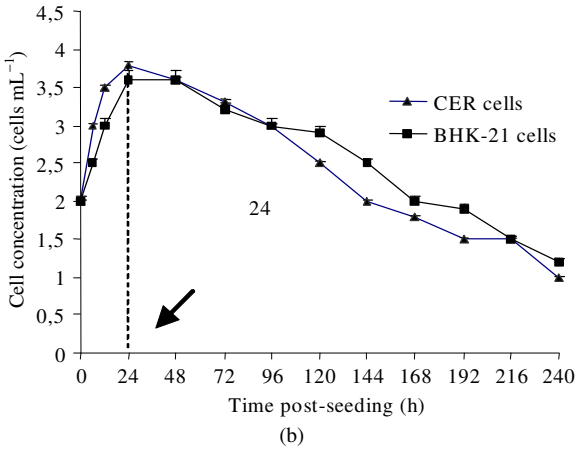


Fig. 1(a-b): (a): BHK-21 and CER cells growth curve obtained after cultured in RPMI 1640 medium; (b): BHK-21 and CER cells growth curve obtained after cultured in MEM+FBS. x-axis = Cell concentration (Log_{10}); y-axis = in different time of culture (hours)

(Fig.1a). The highest cell concentration was found at 48 h after seeding, $3,3\times10^6$ and $3,4\times10^6$ cells mL^{-1}, for CER and BHK_{21}, respectively. As shown, lower surveillance of CER and BHK_{21} cells could be detected, whereas at 48 h post-seeding the amount of viable cells decrease (Fig. 2b).

Susceptibility of CER and BHK_{21} to different strains of IBDV: Both cell lines were susceptible to infection with all IBDV strains, giving rise to efficient replication and increase in the infectious virus titres after 5 passages in each cell (Table 2). The only noticeable difference was the lowest titre observed by infection of CER cells with vvIBDV strain.

Table 2: Titres of infectious virus recovered in each of the three passages of three IBDV strains in BHK_{21} and CER cells grew with RPMI 1640 plus IGF-I medium and conventional MEM plus FBS

		Number of passages				
Viral strain	Cell	1	2	3	4	5
Lukert	CER	1.75	2.50	3.00	4.00	4.00
	BHK_{21}	1.80	4.00	4.50	4.50	3.75
F52/70	CER	3.50	3.95	4.00	4.50	4.50
	BHK_{21}	3.70	4.50	4.75	4.25	4.00
vvIBDV	CER	1.00	1.75	2.00	3.75	4.50
	BHK_{21}	1.75	2.00	2.50	3.50	4.00

Titres are expressed in $\log_{10}$ $TCID_{50}$ 100 μL^{-1}

Quantification of viral IBDV by real time RT-PCR SYBR green technology: Relative amount of virus and β-actin internal control in each sample were determine at 48 and 24h after infection, corresponding to the peak of cell growth curve, performed in triplicate. To determine the specificity of the reaction, the Chicken Embryo Fibroblast (CEF) was included as non-infected cells and for sensitivity the same cells infected by each IBDV strain was applied. Before 48h after infection the viral RNA was determined as illustrated (Fig. 2a, b).

Passages of virulent viruses in embryonated egg or cell culture have been used to produce live IBDV virus vaccines. In addition, IBDV has been adapted to a wide spectrum of cell lines usually maintained by addition of fetal bovine serum, at different work concentrations, as growth promoter[13,14]. Several studies report the IBDV Lukert strain adaptation on serum/protein free media however the vvIBDV (Brazilian genotype) was never adapted to propagate on BHK_{21} and CER mammalian cells[15]. Despite of what the name chicken embryo related may tell about the cell origin, most characteristics of CER cells are acquired from the parental hamster cells, which allow characterize these cells as non-derived chicken line[11].

Considering that CER cells has being reported to be permissive for multiplication of Lukert IBDV strains, the multiplication of F52/70 and vvIBDV strains was assayed using such cells[15]. By analysis of yield of amplifiable viral RNA, the vvIBDV produced≅200 less RNA copies, when the RPMI 1640 plus IGF-I medium was used at five passages detected on both CER and BHK_{21} than observed for MEM plus BFS medium, revealed no differences between these two media. In fact, the titres and yield of amplifiable viral RNA were important to certify that IBDV adaptation produced low level of defective viral particles. Besides, by analysis of virus titres no statistical differences were found among IBDV infectious titres from these three strains adapted in either CER or BHK_{21} cells[11,15]. Furthermore, vvIBDV

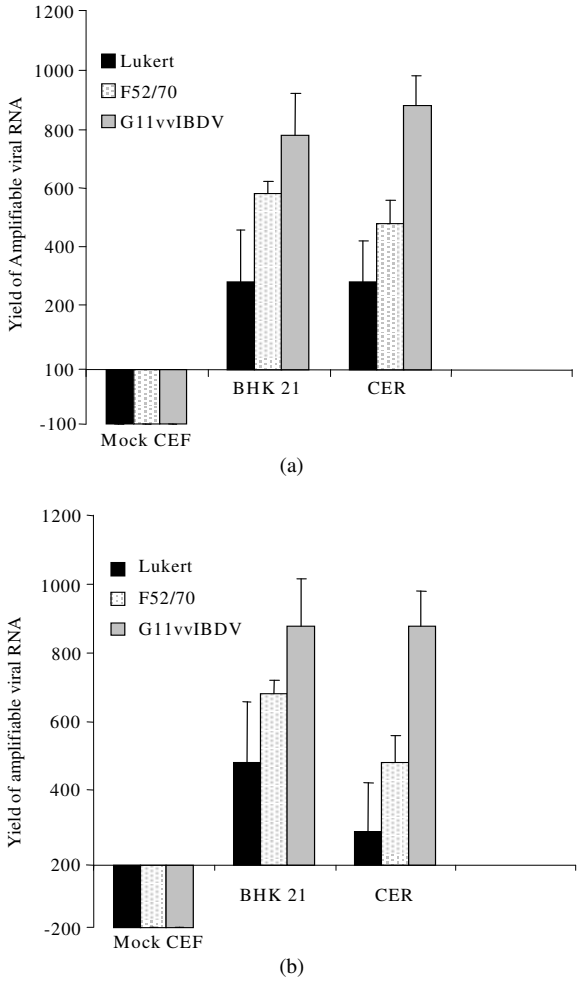


Fig. 2(a-b): Quantification of viral RNA, from the supernatant of infected CEF, BHK_{21} and CER cells by IBDV strains (Lukert, F52/70 and G11vvIBDV) measured by real time RT-PCR SYBR green technology at 48 h post-infection; (a): Growth under RPMI+IGF-1 condition; (b): Growth under MEM+FBS condition at 24h post-infection. Uninfected (mock) chicken embryo fibroblast cell (CEF), infected CEF, BHK_{21} and CER cells at 60h post-infection

has been adapted to growth in Vero-cell line after three serial passages and also attenuated until be non-pathogenic to chicks for vaccine production. These results support the theory that vvIBDV can replicate in cell cultures different from chicken embryo fibroblast and herein it was demonstrated the ability of CER and BHK_{21} supports its replication. It is important to

emphasize that sequences analysis of VP_2 gene of IBDV isolates revealed genetic mutations after adaptation on Vero cells, which were commonly found in the attenuated IBDV strains. The results suggested that virulent field IBDVs could be rapidly adapted to replicate *in vitro* by serial passages in Vero cells and IBDVs adapted to replicate in these cells, also found here for CER and BHK_{21} cells. In spite of no sequence analysis has been performed in the present work, the results found the same ability of CER and BHK_{21} supports vvIBDV replication and, the same way, be able to use as vaccine substrate with no addition of any sera or animal protein[16,7].

CONCLUSION

The use of a safe culture system can stimulate the innovation on biotechnology for vaccine industry. The methodology for CER and BHK_{21} cell proliferation and very virulent IBDV infection could have significant future impact on poultry vaccine industry, but could also to generate significant biological *in vitro* system to understand virus-host interaction.

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