Evaluation of Serum- and Animal Protein-Free Media for the Production of Infectious Bronchitis Virus (M_{41}) Strain in a Continuous Cell Line

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Summary

Infectious bronchitis virus (IBV) is produced in vitro using specific pathogen free chicken embryos, primary chicken kidney cells (CKC) and/or tracheal organ culture (TOC). Regulatory authorities in Europe (EMEA) and in the United States (FDA) have encouraged biological manufactures to reduce or eliminate the use of live animals and/or products of animal origin in biological manufacturing processes. In this paper, a stable chicken embryo-related (CER) cell line was adapted and maintained in serum free (SF) or animal protein free (APF) medium after a direct switch of the medium. The most suitable media were Ex Cell 520 and Ex Cell 302. CER monolayers adapted to SF or APF were infected with IBV (M_{41} strain) in agitated suspended culture and the IBV titre obtained 48 h post infection was 2.8 x 10^4 PFU/ml in both cases. Thus, propagation of CER cells and culture of IBV can be performed without the use of animal serum or animal protein.

Zusammenfassung: Evaluierung von Serum- und Tierprotein-freien Medien für die Vermehrung eines Infektiösen Bronchitis Virus (M41) Stamms in einer permanenten Zelllinie

Infektiöses Bronchitis Virus (IBV) kann in vitro unter Verwendung von SPF-Hühnerembryos, primären Hühnernierenzellen (CKC) und/oder trachealen Organkulturen vermehrt werden. Die Zulassungsbehörden in Europa (EMEA) und in den Vereinigten Staaten (FDA) haben die Hersteller von Biologika aufgefordert, die Verwendung lebender Tiere und/oder Produkten tierischen Ursprungs bei biologischen Herstellungsprozessen zu reduzieren oder ganz zu eliminieren. In diesem Manuskript wird eine stabile Hühnerembryo Zelllinie (CER) beschrieben, die durch einen direkten Wechsel an Serum freie (SF) und Tierprotein freie (APF) Medien adaptiert wurde. Die brauchbarsten Medien waren Ex Cell 520 und Ex Cell 302. An SF oder APF adaptierte CER-Monolayer wurden in gerührten Suspensionskulturen mit IBV (Stamm M_{41}) infiziert, anschließend wurden 48 Stunden nach der Infektion 2,8 x 10^4 PFU/ml in beiden Kulturen gefunden. Die Vermehrung von CER-Zellen und IBV kann also auch ohne die Verwendung von tierischen Seren und Tierprotein erfolgen.

Keywords: IBV, serum free media, animal protein free media, virus production

1 Introduction

Infectious bronchitis virus (IBV) infects the respiratory tract, kidneys and oviduct of chickens of all ages, causing retarded growth, mortality, reduced egg production and inferior egg shell quality (Fabricant, 1998). For the control of virus infection, broilers are usually vaccinated at one day of age with live attenuated vaccines (Cavanagh and Naqi, 1997). In addition, breeders and egg layers are also vaccinated at approximately 8-week intervals with live attenuated vaccines, and with inactivated vaccines after they start laying eggs (Cook et al., 1999). Acute infections are generally diagnosed by immunofluorescence test, ELISA capture assays, virus isolation or serological approaches (Ferreira et al., 2003). Diagnosis of IBV infections by detection of viral RNA by PCR is a fast and specific method (Capua et al., 1999; Cavanagh, 2001; Cavanagh et al., 2002). Virus isolation is performed in specific pathogen free embryos and/or primary cell culture derived from chicks. Nowadays, IBV vaccines are cultured for veterinary use by infecting specific pathogen free chicken embryos via the chorioallantoic route.

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Regulatory authorities in Europe (EMEA) and in the United States (FDA) have encouraged manufacturers of biological products to reduce or eliminate the use of products of animal origin (Sakoda et al., 1998; Butler et al., 2000; Frazzati-Gallina et al., 2001; Kallel et al., 2002). Moreover, culture systems requiring serum are undesirable for the large-scale production of vaccines. Disadvantages of serum supplementation include batch-to-batch variations in composition, high protein content which hinders product purification and the potential for viral, mycoplasma, or prion contamination (Butler et al., 2000).

The chicken embryo related (CER) cell line, which has a heteroploid genome and an antigenic relationship to the BHK21 cell line (WHO Expert Committee on Rabies, 1987), can be used to isolate and replicate rabies virus, infectious bursal disease virus (IBDV) and avian pneumovirus (AVP) (Cardoso and Pilz., 2004; Cardoso et al., 2000; Dani et al., 1999). Recently, it was demonstrated that CER cells support IBV replication (M_{41} serotype) using medium supplemented with foetal calf serum (FCS) (Ferreira et al., 2003).

In this paper, we describe the adaptation of CER cells to two serum free media (SF) and one animal protein free (APF) medium. The kinetics of cell count development were determined in static (T-flasks) and IBV infection was carried out in agitated (spinner) cultures.

2 Materials and methods

2.1 Cell, media and virus

The CER cells were grown as monolayers as described by Ferreira et al. (2003) in Dulbecco's modified Eagle's medium-DMEM (GIBCO/BRL), supplemented with 2 mM glutamine, 10% (v/v) FCS (Sigma), 1 x antibiotic/antimycotic and antimycotic solution (GIBCO/BRL) at 37° C in a moist atmosphere containing 5% (v/v) CO_2. The cells, which were at passage level 25-35, were seeded in 75 cm^2 T flasks (TPP, Switzerland) and subcultured every 2 days. The IBV Massachusetts serotype (strain M_{41}), previously adapted to replicate on CER monolayers, was used (Ferreira et al., 2003). The serum free medium Ex Cell 520 (JRH Biosciences, Lenexa, KS, USA) and the animal protein free media Ex Cell 302 (JRH Biosciences) and Rencyte BHK (Medicult, Jyllinge, Denmark) were used to support CER culture after adaptation and during the IBV infections.

2.2 Multistep CER adaptation in static culture

The CER cells were adapted to SF and APF medium either by following a multistep method or by direct switch of the media in static culture. For the multistep method, the SF and APF percentage was increased progressively (25%, 50%, 75% and 100%), replacing the original DMEM plus FCS medium. The multistep method was performed in T-flasks with 4 x 10^6 cells/ml seeded and the monolayer 90% confluent. The experiments were carried out in triplicate, samples were taken daily for count, glucose, lactate and ammonia concentration and pH measurements.

2.3 CER adaptation by direct switch method in static culture

The CER cells were adapted to SF and APF medium by direct switch from DMEM with 10% FCS. The respective cells were directly transferred to 100% ExCell 302, ExCell 520 or Rencyte BHK as described by Wu et al. (2004), for the development of Enterovirus type 71 vaccine.

2.4 Metabolite analysis and cell counting after CER adaptation in static culture

The glucose and lactate concentrations were monitored by enzymatic assays using specific assay kits (Chronolab, Switzerland, cat # 101-0014, 101-0040). Ammonia was quantified by the enzymatic UV-test (Sigma cat # 171-UV, St Louis, USA). For CER cells growing as monolayers, 0.5 ml of a trypsin/versene solution was added to 0.5 ml of a cell sample. After incubation at 37° C for 10 min, cells were stained with trypan blue (0.2% w/v in PBS) and counted using a hemacytometer. The experiments were carried out in triplicate; samples were taken daily for count, glucose, lactate and ammonia concentration.

2.5 IBV Infection

The IBV infection was performed in spinner flasks (Belco) containing 175 ml of each medium tested, i.e. Ex Cell 302, Ex Cell 520, Rencyte BHK and the standard DMEM plus 5% FCS, placed on a magnetic stirrer at 60 rpm at 37° C at humidified atmosphere with 5% CO_2. Briefly, the adapted CER cells were detached from T-flasks (static culture) using trypsin-EDTA, and then transferred to the spinner flasks at 4 x 106 cells/ml with 2.0 g/L cytodex-1 microcarrier (Amersham Pharmacia). The microcarrier was allowed to settle in the spinner flasks for 3 min. IBV infection was performed by replacing 70% medium with fresh medium containing M_{41} in an amount corresponding to a multiplicity of infection (MOI) of 1, diluted 1:10 and incubated for 120 h. The virus-containing supernatant was dispensed in cryogenic vials 0.5 ml (Corning) volumes and stored at -80° C until needed.

2.6 Determination of virus titre

Plaque assays were undertaken with monolayers in 6-well culture plates (TPP), according to Cardoso et al. (2004). Each well received 4 ml cell suspension with 4 x 10^6 cells/ml in DMEM plus 10% FCS and plates were incubated for 24 h at 37° C in a humidified CO_2 atmosphere. The growth medium was aspirated from each well, and 0.5 ml of virus suspension was added to at least three wells each. 1:10 dilutions were performed using PBS. The plates were rocked gently immediately after inoculation and once more about half way through the incubation period of 90 min at 37° C in a humidified atmosphere. The overlay medium, which consisted of equal volumes of 2 x media and 2 x 4% sephadex G-100, was prepared as described before (Bussereau et al., 1982) during the virus adsorption period.

Sephadex was dissolved at 4% (w/v) in 100 ml deionised distilled water and was sterilised by autoclaving for 45 min at 121° C. The solutions were then kept in a water bath at 43° C. The final overlay medium was prepared by mixing equal volumes of the 2 x media and the 2 x sephadex solution, and at the end of the virus incubation period, each well received 4 ml of the solution. The plates

were left at room temperature for 20 min, and then incubated at 37°C for 72-96 h in a humidified 5% CO_2 atmosphere. The monolayers were fixed for a minimum of 4 h by adding 2 ml per well of a 3.7% solution of formaldehyde in physiological saline (MERCK, Brazil). The formaldehyde was discarded and the sephadex overlay plugs were removed by holding the cell culture plate at a 45° angle under a gentle stream of cold running tap water. The cell culture plate was then tapped gently upside down on a paper towel to remove the plugs and drain off the water. The fixed cell monolayers were stained with 0.1% (w/v) crystal violet solution for 20 min. Plaques were counted, characterised and the virus infectivity titre expressed as plaque forming units (PFU) per ml.

2.7 Statistical analysis

The results are given as mean ± S.D. (standard deviation) and all experiments were repeated three times. The Graphpad Prism version 3.00 for windows was used to performed all the graphics (Motulsky, 2004).

3 Results and discussion

The aim of this study was to investigate whether CER cells could be adapted to culture in SF and APF media and to study whether cells grown in these media are still conducive to IBV infection.

Adaptation of cell lines to SF and APF media have been achieved successfully for BHK cells (Cruz et al., 1998; Merten et al., 1999), MDCK cells (Kessler et al., 1998) and C13, BSR and Vero cells (Merten et al., 1999).

For the CER cells, multistep adaptation to SF or APF media was not feasible (Tab. 1). When the cells were transferred to 75% Rencyte BHK, cell aggregates formed. At 75% ExCell 520 or ExCell 302, the CER cells grew in suspension as small clumps and died after a few days.

Regarding the direct switch of DMEM plus FCS to SF or APF media, cells were adapted successfully to both Ex Cell media but not so well to Rencyte BHK medium (Tab. 1). CER cells were considered totally adapted when confluence reached 80% and cells did not form clumps. The culture underwent at least five passages in the new media before the kinetic studies were performed. Cells growing in Rencyte BHK did not form a confluent monolayer. Hence, cells grown in Ex Cell 302 and 520 were used in the following experiments. For comparison, although BHK cells have been adapted successfully to some protein-free media by direct switch, adaptation to Ex Cell 302 medium was not successful (Kallel et al., 2002).

Tab. 1: Adaptation of CER monolayers to SF and APF media without IBV infection in static culture.

	Medium		
	Rencyte BHK	Ex Cell 302	Ex Cell 520
multistep adaptation	no	no	no
direct switch	no	yes	yes

As shown in Figure 1 (A and B) CER cells exhibited similar growth behaviour in both Ex Cell media studied. Cell density dropped initially, and then remained stable until 100 h in Ex Cell 302 (APF) and Ex Cell 520 (SF) media respectively. During this time, glucose was used up and lactate was produced steadily while ammonium concentrations remained stable. The higher 8concentration of glucose (25m M) in Ex Cell 520 might explain why the initial drop in viable cells was greater in this medium. Similar find-

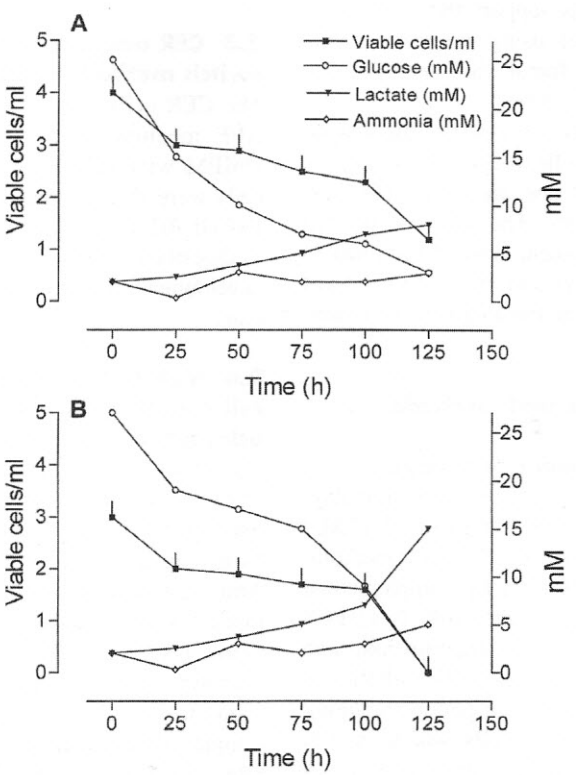

Fig. 1: CER growth curve in static culture in (A) Ex Cell 302 (APF) and (B) Ex Cell 520 (SF) media without IBV infection. Concentrations of glucose, lactate and ammonia in the medium are shown.

ings were reported by Kallel et al. (2002), and the glucose concentration was higher than inhibitory concentrations reported for BHK cell growth (Butler and Spier, 1984, Cruz et al., 2000).

IBV infection was performed to ascertain virus replication under these new cell culture conditions: new media and agitated suspended culture. In both media, the secretion of IBV coincided with a peak of cell density (Fig. 2 A and B). Moreover, a maximal virus titre of 2.8 x 10^4 PFU/ml was obtained 48 h post infection, 24 h earlier than observed in cells grown in DMEM +5% FCS (Fig. 2A and B) in both Ex Cell media, independent of their different composition. This observation indicates that some components in the FCS may affect the viral assembly and release; however this must be investigated further. Finally, the satisfactory IBV titre found at 48 h post infection indicates that both media are suitable for IBV production by infecting CER cells in suspension. Nevertheless, it must be observed that IBV titres are still lower than those reported for chicken embryo infection, CKC and TOC (Cook et al., 1999).

Efforts toward standardisation of cell culture protocols in Good Cell Culture Practice are continuing (WHO, 1987). In addition, considerable ethical concerns were raised recently about the collection of FCS. Thus, in order to decrease the annual need for bovine foetuses, improvement of cell and culture methodology using serum-free or animal protein free medium represents a valuable scientifically well accepted alternative to the use of animal protein for virus propagation. Finally, the use of serum free and animal protein free media to propagate CER cells infected with IBV can be a safe and useful laboratory technique, with a considerable economic and ethical impact.

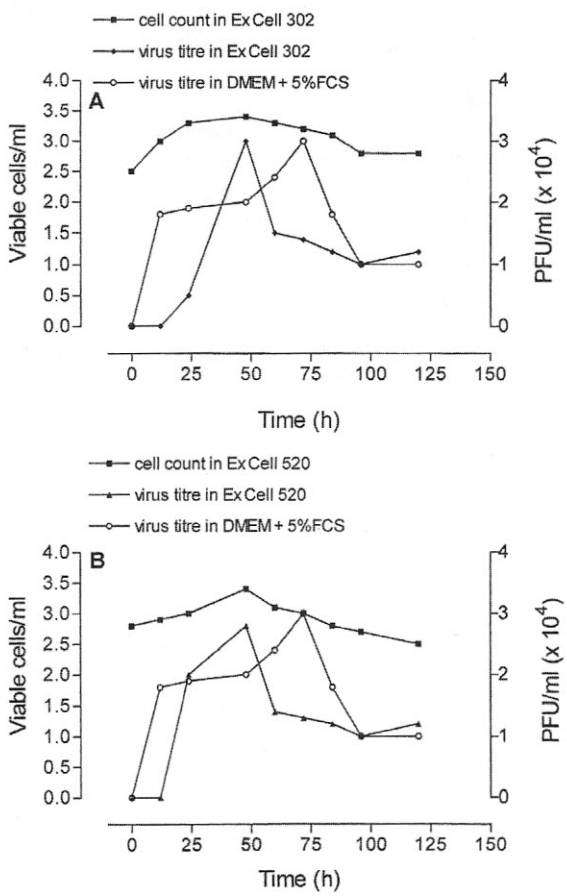

Fig. 2: Comparison between virus titres (PFU/ml) induced in CER cells grown in Ex Cell 302 and DMEM plus 5% FCS (A) and in cells grown in Ex Cell 520 and DMEM plus 5% FCS (B) in agitated culture.

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