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Pathogenicity, Antigenicity and Immunogenicity of Infectious Bursal Disease Virus (IBDV) Due to Propagation in Chicken Embryo Related Cell Line Using Serum-free Medium

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Abstract: Relative pathogenicity, antigenicity and immunogenicity of standard Lukert strain of IBDV were observed in Chicken Embryo Related (CER) cell maintained in Ex Cell 520 serum free medium following adaptation. It also used Lukert strain of IBDV propagated in Bursa of Fabricius (BF), chicken embryos and Chicken Embryo Fibroblast (CEF). Bursa derived-IBDV induced the most severe lesions in BF when compared with those propagated in CEF and CER cells, which produced mild lesions. Antibody induced by cell culture-derived IBDV neutralize bursa and embryo derived-IBDV less effectively, whereas antibody induced by CER-derived virus neutralize well CEF-derived IBDV. Moreover, after 14 days of IBDV infection, CEF and CER-derived IBDV produced same level of antibodies. A high frequency of virus re-isolation (obtained from bursae, spleen, thymus and bone marrow of infected birds) was observed by bursa and embryo derived-IBDV at all p.i. However the viral RNA detected by RT-PCR showed positive results for all negative ones in virus re-isolation. These results document that the relative pathogenicity, antigenicity and immunogenicity is not reduce significantly following IBDV adaptation in CER cells, using serum free medium, compared with those obtained by CEF-derived IBDV.

Key words: IBDV, CER replication, Pathogenicity, Immunogenicity, Antigenicity, Serum free medium

Introduction

Infectious Bursal Disease Virus (IBDV) belongs to *Avibirnavirus genus* and is considered the causative of an important and acute contagious disease of poultry industry and has spread worldwide. Two serotypes (I and II) can be differentiated by cross-neutralization assay and ELISA using monoclonal antibodies (Nick *et al*., 1976; Becth *et al*., 1988 and Kibenge *et al*., 1988). Serotype I IBDV strains are pathogenic for chickens and can cause serious problems in the poultry industry, however individual strains differ markedly in their virulence (Yamaguchi *et al*., 1996).

Since the mid 1980's a new IBDV serotype I pathotype, 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 (McFerran *et al*., 1980, van den Berg *et al*., 1991, Eterradossi *et al*., 1999 and Di Fabio *et al*., 1999). In general, the IBDV whose genome consist of double strand RNA (segments A and B) has actively dividing B lymphocytes as target cells for its replication. The smaller segment (segment B) ecodes VP_1, a 90-kDa protein, which correspond to RNA-dependent RNA polymerase. Consequently, the major segment A, containing two open reading frame (ORFs) ecodes a precursor protein, processed into three mature viral polypeptides, VP_2, VP_4 and VP_3. The respective amplification of VP_2 gene sequence has been used on reverse-transcription/polymerase chain reaction (RT-PCR) in molecular diagnostic (Tham *et al*., 1995, Cavanagh 2001, Zhang *et al*., 2002 and Barlic-Maganja *et al*., 2002).

The role that different propagation methods have in modifying the pathogenicity of IBDV has been demonstrated that viruses passaged in birds maintain their pathogenicity, whereas viruses propagated in embryos may maintain or lose their pathogenicity. The effect of host systems on the antigenicity and/or immunogenicity of IBDV suggest the importance in the propagation of IBDV for vaccine preparation, protecting the flocks against a challenge in the field (Rodriguez-Chaves *et al*., 2002).

Relevant to IBDV serotype I detection, the viral infection leads to destruction total or partial of bursa of Fabricius follicles and the respective histopathologic examination has been used as diagnostic. Serotype I strains adapted to tissue culture by serial passaging showed reduced in vivo virulence in infected chicken (Yamaguchi *et al*., 1996 and van Loon *et al*., 2002). However, more recently was described the adaptation to tissue culture of vvIBDV strain by site-directed exchanging of certain amino acids using a reverse genetics system and the influence on virulence was assayed by animals experiments, demonstrating that VP_2 plays a decisive role in the pathogenicity of IBDV (van Loon *et al*., 2002).

Actually, attempts have been made to develop techniques for IBDV detection and new cell lines have been proposed for its isolation and replication In addition, the classical IBDV strain has been adapted to replicate and produce Cytophatic Effects (CPE) in primary cell cultures, including chicken bursa lymphoid cells, chicken embryo kidney cells and chicken embryo fibroblast cells. Furthermore, mammalian continuous cells lines would be more suitable for IBDV isolation and Baby Grivet Monkey cell (BGM70), Vero cell and Chicken Embryo Related (CER) cell line have been used for this purpose (Lukert *et al*., 1974, Jackwood *et al*., 1986 and Cardoso *et al*., 2000). In fact, standard cell proliferation step used various animal sera to enhance cell growth (Kallel *et al*., 2002). However, supplementing cell culture media with such components presents several drawbacks like lot to lot variation, potential risk of contamination by viruses, mycoplasma, prions, etc. Moreover high quality sera are particularly expensive (Reculard, 1996).

This study was conducted to assess the differences in the relative pathogenicity, antigenicity and immunogenicity of IBDV (Lukert strain) adapted and propagated on Chicken Embryo Related cell (CER) using serum free medium. For this purpose, Lukert strain was propagated also in different host system, birds, embryos and primary cell culture and the results compared with those obtained by replication on CER cells propagated in Ex Cell 520 serum free medium.

Materials and Methods

Cells: Suspensions of Chicken Embryo Fibroblast (CEF) were prepared as confluent monolayers for in vitro propagation of IBDV strain and microneutralization assays. CEF were prepared from 10-day-old specific pathogen free (SPF) chicken embryos (Granja Rezende, Uberlandia, Brazil) as previously described (Cardoso *et al.*, 1998). Cell concentrations were adjusted to 5 x 10^5 CEF/mL and plated in tissue culture flasks (20 mL CEF/T_{75} cm^3 or 40 mL CEF/T_{150} cm^3) for virus growth or in 96-well flat-bottomed plates (100 µL CEF/well) for microneutralization assays. Confluent CEF monolayers were formed after 24h incubation at 37°C with 5% CO_2.
Chicken embryo related (CER) cell line was cultured according to Cardoso *et al.*(2000) and Ferreira *et al.* (2003). The cells were obtained from Dr Clarice Arns provided by Justus Liebig Universität, Gissen, Germany, maintained in our laboratory from passage 35 to 45.

Virus: The IBDV strain Lukert obtained from Intervet Laboratories (Campinas, Brazil) was used after propagated in different host systems (Rodriguez-Chavez *et al.*, 2002). The virus was propagated in SPF chickens before arrive in our laboratory.

Culture medium and Virus Production in CER Cells: The following medium Ex Cell 520 (JRH Biosciences, Lenexa, KS) was used as recommended by Kallel *et al.* (2002) to propagated Baby Hamster Kidney (BHK-21) cells. Cell culture assay was performed at 37°C, in T_{75} cm^3 flasks, at a concentration of 3 x 10 ^{6}cells /100 µL. Samples were taken daily in duplicate to determine cell concentration. The CER cells were infected by Lukert strain at a cell concentration of 1 x 10^6 cells /100 µL with a multiplicity of infection (MOI) of 1.0 . Virus production was performed at 37°C, in T_{75} cm^3 flasks and the samples taken in duplicate daily to determine cell concentration and virus titer.

***In vivo* Propagation of IBDV:** The Lukert strain was propagated once in SPF Leghorns, gently provided by National Reference Laboratory-LANARA (Campinas, Brazil). Five groups of 5-week SPF Leghorns were wing-banded and placed immediately in GPM-1200 glove-port isolator units that contained HEPA filters in the intake and exhaust. It was used 5 birds infected with IBDV at 10^4 median embryo infectious dose (EID_{50}) 0.2 of virus ($10^{3.7}$ intraocularly and $10^{3.7}$ intranasally). Four days post-innoculation (p.i) bursae were collected and a 20% (w/v) bursal homogenate was prepared in phosphate-buffered saline (PBS) with antibiotics as described before (Cardoso *et al.*, 1998). Homogenates were clarified by centrifugation at 400 x g for 10 min at 4°C. The supernatant containing IBDV (Lukert strain) bursa-adapted was aliquoted and stored at –70°C. The same procedure was followed to process the uninfected 20% bursal homogenates.

***In Ovo* Propagation of IBDV:** The original IBDV Lukert strain was submitted to 10 serial passages in 10-day-old SPF embryonated chicken eggs as follow. SPF embryos were inoculated with IBDV bursa-adapted suspension by the chorioallantoic membrane (CAM) route using 10^4 EID_{50}/embryo as described by Rodriguez-Chavez *et al.* (2002). The final dose of IBDV inoculated was 10 EID_{50}/0.1 mL. The CAM was harvested, pooled and weighed at 6 days (p.i.) and CAM pools were diluted 1:1 (w/v) in PBS with antibiotics, homogenized using a blender, frozen (-70°C) and thawed five times. The homogenates were centrifuged at 400xg for 10min at room temperature and the virus-containing supernatant was collected, aliquoted and stored at –70°C. The same procedure was followed to prepared uninfected CAM homogenates. Embryos in the uninfected group were inoculated with 0.1 mL of PBS with antibiotics via CAM route.

***In vitro* Propagation of IBDV:** The original IBDV Lukert strain was passage 10 times in CEF and CER cells monolayers (Cardoso *et al.*, 2000).
The CEF monolayers were inoculated by adding 10^5 PFU (plaque unit forming) of IBDV directly to the growth medium. The IBDV-infected CEF cells were incubated for 2 to 4 days, the cytopathic effect (CPE) assessed daily and cells and supernatant harvested when approximately 50% CPE was observed. The cells and supernatant were frozen (-70°C) and thawed five times. The samples were clarified by centrifugation as described before. Plaque assay was performed as described by Villegas (1998). Non-infected CEF cells were harvested and processed in parallel as described for infected CEF cells.
The CER monolayers were inoculated using 0.5 mL of the original stock (Lukert strain propagated on birds) in 1 mL Dulbeccos modified Eagles medium (DMEM), antibiotics without foetal calf serum.. The amount inoculated was 10^5 PFU and IBDV- infected CER cells were incubated for 2 to 4 days. The CPE was assessed daily and cells and supernatant harvested when approximately 50% CPE was visualized.(Cardoso *et al.*, 2000).

Titration by Quantal Assay: Virus titrations were performed on each bursa-derived, embryo-derived and cell culture-derived IBDV by inoculating 10-fold serial dilutions (10^{-1} to 10^{-10}) via CAM route into 10-day-old SPF embryonated eggs (Villegas, 1998). Virus dilutions were prepared in PBS with antibiotics and 0.1 mL of inoculum was injected per embryo. Four embryos were used per dilution. Embryos were incubated for 6 days and candled daily to check for mortality. Lesions were recorded after performing examination of embryos that were previously chilled at 4°C overnight. Non-specific mortality within the first 48 h was not included in the calculation for virus titre, which was determined for each IBDV seed stock by Reed and Muench (1938) method and expressed as EID_{50}/mL.

Chicken Polyclonal IBDV Antisserum Preparation: Chicken polyclonal IBDV antiserum was prepared separately for each bursa-derived, embryo-derived and cell culture-derived Lukert strain using 5-week-old SPF Leghorns. To produce antibody against the bursa-derived IBDV, 5 chickens were used. A dose of 10^4 EID_{50}/0.2 mL per bird was administered ($10^{3.7}$ intraocularly and $10^{3.7}$ intranasally). The control birds received 0.2 mL of uninfected bursal homogenate. Chickens were held for 3 weeks in isolation, bled and serum collected. A similar procedure was used for production of serum against the embryo-derived IBDV. To produce serum against the both cell culture-derived IBDV (CEF and CER) 10 chickens were used. Birds were wing-banded, separated into 2 Groups of 5 birds and placed immediately into isolator units. Cell culture-derived IBDV was inoculated, both of them, subcutaneously (10^5 PFU/0.2 mL per bird) except for the uninfected group, which received 0.2 mL of CEF and CER growth medium subcutaneously. Inoculated birds were held for 3 weeks, bled, antisera collected from each group, heat-inactivated at 56°C for 30min and assayed for antibodies against potential contaminating agents (chicken anemia and reovirus).

Microneutralization Assay: This was performed according to Rodriguez-Chavez *et al.* (2000). The standard strain from Bursavac 4 (BV4),

cell cultured- Lukert derived IBDV, was titrated and diluted in M199 medium with antibiotics to yield 1000 plaque forming units (PFU) per 25 µL. This strain dilution was added for all the testing plate. Four-fold serial dilutions were prepared for each antiserum (1 :4 to 1 :65 536) in a final volume of 75 µL of M199 medium with antibiotics using 96-well plates. Each antiserum was recorded as reciprocal of the highest dilution where monolayers remained 100% intact compared with the control wells. The geometric mean titre (GMT) of triplicate samples was calculated for each antiserum (Villegas, 1998). The assay was conducted three times and a final GMT was determined.
Sera prepared against bursa-derived, embryo-derived and cell culture-derived IBDV (Lukert strain) were evaluated by microneutralization assays to normalize the antibody concentration versus the standard BV4 strain. Antisera that had a neutralization titre of 1 :128 were pooled, aliquoted and stored at –20°C, as recommended by Rosenberger *et al.* (1998).

***In Ovo* Neutralization Assay:** VN assays were carried out in 10-day-old, SPF embryonated eggs using chicken polyclonal antiserum prepared against bursa-derived IBDV, embryo-derived IBDV and both cell culture-derived IBDV (Lukert strain). Each sample was diluted separately in PBS with antibiotics and tested against their homologous and heterologous sera of the same source. The procedure and respective results analysis was carried out as described by Rodriguez-Chavez *et al.*, (2000). A neutralization index (NI) of 0 to 0.9 log units is regarded as negative, a NI of 1.0 to 1.69 units as equivocal and NI of 1.7 log units or greater as positive. The percentage relatedness (*R* value) between the different host adaptation of IBDV was interpreted by <10% is considered to have another serotype, 10 to 31% is considered to have major subtype differences, 32 to 70% is considered to have minor subtype differences and 71 to 100% is not considered different (Lukert, 1991).

Pathogenesis Study: Twenty five 4-week-old SPF Leghorns were obtained from Granja Rezende (Uberlandia, Brazil) and 4 chickens from this flock were bled (0.5 mL of blood per bird, day 1) and serum harvested for antibody assays. Chickens were divided in five groups of five birds, identified and placed as described previously. The birds were inoculated 10^4 EID_{50} ($10^{3.7}$ intranasally and $10^{3.7}$ intraocularly) of Lukert strain propagated in birds (Group 1), embryos (Group 2) and both cell line (CEF Group 3 and CER Group 4). The last five birds were used as control. The birds were bled and sacrificed at 2, 4, 6, 10 and 14 days p.i. Individual lymphoid tissue samples were collected from each bird for microscopic examination, virus re-isolation and viral RNA detection (bursa of Fabricius, spleen, thymus and bone marrow). Respective sera were obtained from blood samples taken at each sampling (including day 1). The sera were heated at 56°C for 30 min and stored at – 20°C until analyzed. Relative IBDV pathogenicity was based on signs, gross and microscopic lesions, IBDV re-isolation and serological findings as assessed by enzyme-linked immunosorbent assay (ELISA) (Flock-Check IBD ELISA kit ; Idexx Laboratories INC., Westbrook, ME, USA). These titres were expressed as the arithmetic mean ± the standard deviation of the antibody titre calculated.

Virus Re-isolation: A 20% pooled tissue homogenate was prepared in PBS with antibiotics for each lymphoid tissue type (bursa of Fabricius, spleen, thymus and bone marrow). Each homogenate was inoculated into five 10-day-old SPF embryonated eggs (0.1 mL per embryo) via CAM route, as described before (Cardoso *et al.*, 1998). Embryos were incubated at 37°C with 55% humidity for 6 days. Mortality was observed daily. Dead embryos (non-specific mortality observed within 48h p.i.) were discarded. After 6 days, embryos were chilled at 4°C overnight and examined for standard strain-induced lesions, including stunted haemorrhagic embryos with parboiled livers and also embryo mortality (Rodriguez-Chavez *et al.*, 2000).

Microscopic Examination: Pooled bursa samples were fixed in 10% (v/v) neutral buffered formalin for a minimum of 24h, processed, embedded and cut in 6μm thin sections. The slides were stained using a standard haematoxylin and eosin staining programme. After the staining, each slide was dipped in xylene, mounting and cover-slipped. Tissue sections were examined under a light microscope and lesions recorded (Tanimura *et al.*, 1995 ; Rodriguez-Chavez *et al.*, 2000c).

Reverse Transcriptase Chain Reaction (RT/PCR): The respective suspension of bursae, spleens, thymus and bone marrow were submitted first to a digestion using lysis buffer containing PBS 0.5M NaCl and 20 µg mL^{-1} proteinase *K* for overnight at 4°C and after the RNA extraction was performed using Trizol® reagent (Cardoso *et al.*, 2000). The extracted viral RNA was precipitated using ethanol and resuspended in 90% dimethyl-sulfoxide (DMSO). The precipitated RNA was re-suspended in 100 µL DMSO solution and incubated at 98°C for 5 min. Brief, 1 µL of RNA in DMSO was then amplified by RT-PCR using the One Step RNA PCR Kit (Invitrogen, Life technologies) according to the manufacture's instructions. One set of primers were used (A3.1 sense 5'-GATTGTTCCGTTTCATACGGA-3' and A 3.2 antisense 5'-AGTGTGCTTGACCTCACTGT-3') were previously described (Tham *et al.*, 1995). The RT-PCR sing these primers amplifies a 309-bp of conservative region on VP_2 for IBDV serotype 1. The amplicons were observed by electrophoresis in 1.5% of agarose gel with 0.5µg mL^{-1} ethidium bromide. The specificity of RT-PCR was performed suing non-infected bursae samples and the RNA extracted from bovine rotavirus. IBDV RNA extraction was performed from all the tissue samples that were negative for virus re-isolation in embryos inoculation. Total RNA were prepared from infected tissues (bursa of Fabricius, thymus, spleen and bone marrow) obtained by infection with IBDV-bursa, embryo and cell culture derived virus. The respective RNA was extracted by Trizol LS® method following the manufactures instructions. Purity of the RNA was analysed by 1% agarose gel electrophoresis and ethidium bromide staining. Prior to cDNA synthesis, 5 µL of RNA were denatured at 65°C for 15min and at 98°C for 3min followed by quick chilling on ice water. Random hexamers (30ng/ µL) were used to prime the cDNA synthesis by SuperScript II Rnase H-reverse transcriptase (BRL, Invitrogen, CA, USA) in a 20 µL reaction. The sythesis was performed at 42°C as described by manufacturer. After synthesis, cDNA preparations were diluted by addition of 40 µL water. Then 5 µL of cDNA preparations were used in the PCR amplifications by OneStep® procedure. The oligonucleotides used in the PCR amplification were those described by Tham *et al.* (1999).The amplifications were performed in a thermocycler and the products obtained by amplification visualized by 1% agarose gel electrophoresis and ethidium bromide staining.

Statistical Analysis: The statistical significance of the differences among groups was determined by the Mann-Whitney (two-tailed) test. P<5% was considered to be statistically significant. The results are reported as mean ± standard deviation.

Results and Discussion

The CER cells propagated in Ex Cell 520 serum free medium presented the same ratio, proliferation and metabolism as observed by traditional foetal calf serum supplement procedure (data not shown).

Virus neutralization experiment was conducted in embryonated eggs to assess the antigenic and immunogenic variation of Lukert strain (IBDV serotype 1) after propagation in birds, embryos and cell culture. These results are shown in Table 1 and 2, where the NI and the corresponding calculated *R* values are expressed respectively.

The results show that neutralization ability of the normalized antisera changed depending on the virus propagation and the strain-antiserum combinations included in the VN assay. NIs were generally higher for the homologous strain-antiserum. Furthermore, sera prepared against CER-derived IBDV neutralized the CEF-derived IBDV and demonstrated poor capacity to neutralize bursa and embryo-derived IBDV. The calculated *R* values showed that CER adaptation suggest major subtypes differences (21% and 31%) with bursa and embryos-IBDV sources and minor subtype differences with CEF-derived IBDV (50%).

Birds inoculated with Lukert strain bursa-derived developed typical clinical signs of the disease after a 3 days of incubation. The clinical signs observed included depression, reluctance to move, poor feed and water intake, watery diarrhoea, prostation and dehydration. It was not observed the same clinical signs in birds inoculated with embryo or cell culture adapted IBDV. Microscopic assessment of lymphoid organs (bursa of Fabricius, thymus, spleen and bone marrow) only revealed gross lesions in bursae of birds inoculated with bursa and embryo-derived IBDV. However mild lesions were also observed from CER-derived IBDV bursae sections and characterized by diffuse follicular lymphocyte loss in association with a minimum seroheterophilic infiltration (data not shown).

Patterns of virus re-isolation are expressed on Table 3. The virus was not re-isolate from thymus (10 and 14 days p.i.) and bone marrow (6, 10 and 14 days p.i.) in birds inoculated with bursa and embryo-derived IBDV. However, from birds inoculated with embryo-derived IBDV in spleen at 6 and 10 days p.i. no virus could be isolated also. Relevant to IBDV cell adaptation, CEF-derived virus was not re-isolated from bursa and spleen (10 and 14 days p.i.), thymus and bone marrow (6, 10 and 14 days p.i). The major difference observed with CER-derived IBDV in re-isolation assay, was the positive results obtained from early collected points :bursa day 2 and 4 p.i. ; spleen at days 2, 4 and 6 p.i. ; thymus and bone marrow at day 2 p.i. Otherwise, RT-PCR results were positive for all samples tested, which presented negative results in re-isolation approaches.

Seroconversion of chickens inoculated with IBDV adapted in different host systems is shown in Fig. 1. ELISA results showed that both cell adapted IBDV induced the same level of antibodies, with high titres at day 6 p.i. However, IBDV propagated in birds or embryos

Table 1: Assessment by in ovo neutralization assay in 10-day-old SPF embryos of the antigenic and immunogenic variation of Lukert strain propagarted in different host systems (bursa of Fabricius, embryo, or cell culture)

	Inoculum[a]	
Source	Antiserum	Neutralization Index[b]
IBDV bursa-derived IBDV bursa-derived		5.00
	IBDV embryo-derived	3.17
	IBDV CEF-derived	1.26
	IBDV CER-derived	0.35
IBDV embryo-derived	IBDV bursa-derived	3.70
	IBDV embryo-derived	3.12
	IBDV CEF-derived	2.46
	IBDV CER-derived	2.44
IBDV CEF-derived IBDV bursa-derived		3.80
	IBDV embryo-derived	3.76
	IBDV CEF-derived	4.00
	IBDV CER-derived	4.00
IBDV CER-derived IBDV bursa-derived		1.76
	IBDV embryo-derived	3.98
	IBDV CEF-derived	4.00
	IBDV CER-derived	4.00

a- Ten-fold serial dilutions of virus were prepared and combined with either homologous or heterologous normalized (VN = 1 :128 against BV4) IBDV standard serum obtained by Intervet Laboratories

b- The NI was calculated as described in Materials and Methods

Table 2: Percentage antigenic relationship (R values) between Lukert strain propagated in different host systems (bursa of Fabricius, embryo, or cell culture)

	Bursa-derived[a]	Embryo-derived	CEF-derived	CER-derived
Bursa-derived	100[b]			
Embryo-derived	98	100		
CEF-derived	60	84	100	
CER-derived	21	31	50	100

a- Host system b- Antigenic relatedness *R* value calculated as described in Materials and Methods section

Table 3: Re-isolation of Lukert strain propagated in the bursa of Fabricius, embryo and cell culture

	Tissue sample homogenates collected[a] on specific days post-inoculation																			
	Bursa[b]					Spleen					Thymus					Bone marrow				
Source	2[c]	4	6	10	14	2	4	6	10	14	2	4	6	10	14	2	4	6	10	14
Bursa-derived	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	-	-	-
Embryo-derived	+	+	+	+	+	+	+	-	-	+	+	+	+	-	-	+	+	-	-	-
CEF-derived	+	+	+	-	-	+	+	+	-	-	+	+	-	-	-	+	+	-	-	-
CER-derived	+	+	-	-	-	+	+	+	-	-	+	-	-	-	-	+	-	-	-	-
Controls[d]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	

a- 20% tissue type homogenate prepared from pooled samples that were collected at specific days post-inoculation

b- Lymphoid tissue collected from five birds c- Days post-inoculation

d- IBDV was re-isolated in embryos; -, IBDV was not re-isolated in embryos

induced high antibody response observed on all days p.i ; comparing to those obtained from cell culture adapted IBDV.

In this paper, we describe the adaptation of CER cells to serum free medium Ex Cell 520 and also IBDV production by infected cells carried out in static cultures. The CER cell line has been frequently used to isolate and also replicate rabies virus, being described in World Health Organization proceedings as having antigenic relationship to the BHK-21 cell line. In the same way, BHK-21 has been well propagated in Ex Cell 520 medium, which encouraged the present study (Smith *et al*., 1977, Kallel *et al*., 2002, WHO Expert Committee on rabies, 1992). In general, during the standard cell proliferation step, various animal sera are used to enhance cell growth, which are

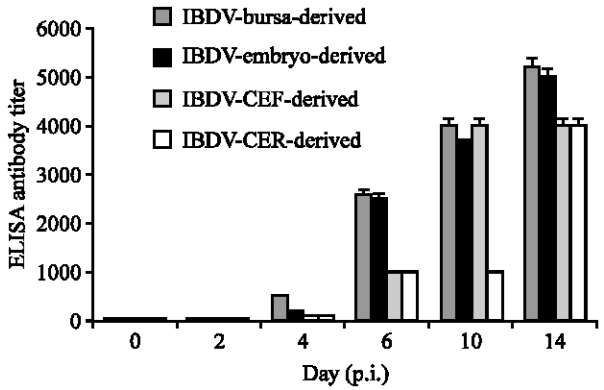

Fig. 1: Antibody titers of Lukert IBDV strain propagated in bursa of Fabricius, embryos and cell culture by FlockCheck IBD ELISA kit

particularly expensive and should be checked frequently. Actually, current biotechnological processes for the production of biological omit the use of serum supplemented media. Furthermore, regulatory authorities in Europe (European Medicine Evaluation Agency: EMEA) and in the United States (Food and Drug Administration: FDA) have encouraged biological manufactures to reduce or eliminate the use of substances of animal origin in their manufacturing process (Castle and Robertson, 1999).

The immunogenicity results showed changes in relative susceptibility of IBDV neutralizing antibodies were related to the source of inoculum used to prepare IBDV antisera. Virus neutralization titres for bursa and embryo derived IBDV antisera were higher than those for cell culture (CER and CEF) derived antisera. These findings is in agreement with a number of other studies testing IBDV host adaptation. (Rodriguez-Chavez *et al*., 2002a, b and c). It was shown that IBDV grown in CER cells were less invasive/pathogenic than embryos or bursa derived IBDV. The both cells studied presented the same results compared with those obtained in vivo, however CEF seems to be more genetic related to chickens and respective embryos than CER cells. Additionally, CER are continuous cell line, easy to propagated when compared to CEF primary system, which contributes to optimize the virus growth.

The present study also showed that CER-derived IBDV showed poorer immunity when compared with response developed by birds infected by bursa or embryo-derived IBDV. Similarly, the capacity of IBDV adapted in different hosts to elicit a protective humoral response, was demonstrated here by differences in normalized NIs and the calculated *R* values. Likewise, it was shown minor subtype difference between CER and CEF- derived IBDV and major subtypes differences between CER- derived IBDV and embryo or bursa-derived IBDV. Furthermore, birds inoculated with Lukert strain derived IBDV do not present clinical signs and mild lesions in the bursa tissue were observed. It was demonstrated also that CER-derived IBDV showed lower immunity when compared with response developed by birds infected by bursa or embryo sources and same levels as observed from CEF-derived IBDV

Interestingly, it was showed here that CER and CEF-derived IBDV induce the same level of antibodies at day 6 and 14 p.i. detected by ELISA. These could be explained by the minor subtype difference and by the higher virus titres obtained after adaptation. Moreover, at late periods p.i. no virus would be isolated from lymphoid suspensions (bursa, spleen, thymus and bone marrow) from birds inoculated with CER-derived IBDV, the respective viral RNA was detected at all p.i. These results may suggest that IBDV attenuation reduce the virus virulence, but also allow low rates of replication, which can be explain the infection of contact birds as observed by others studies (Rodriguez-Chaves *et al*., 2002c). Regarding to pathogenecity, antigenicity and immunogenicity findings, the IBDV replication in cell cultures should be done carefully, especially when live vaccines are used in the field.

We have previously shown that IBDV Lukert strain replicates in CER cells, as the same level as in CEF (Cardoso *et al*., 2000). This system was also used for IBDV field strains (non vvIBDV strains) isolation and the results compared with Vero cells system. (Cardoso *et al*., 2001, Ferreira *et al*., 2004). Additionally, the results presented here point out the similarity between CEF and CER-derived IBDV pathogenicity,

immunogenicity and antigenicity, which could improve the use of these cells in virus production and also as a candidate for low virulent cell adapted vaccine studies, with the advantage of use serum free medium for cell propagation..

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