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ABSTRACT

Susceptibility of chicken embryo related cells (CER) to non-classical IBDV was compared with Vero cells for the same purpose. It was used 20 bursal samples collected from clinical cases of IBDV, positive by histopathological evaluation. After three times infection of CER and Vero cells monolayers, at 6, 12, 18, 24, 48, 60 and 72h post-infection (PI), extra-cellular and intracelluar IBDV was measured. The extra-cellular amount of IBDV particles was quantified by DAS-ELISA from 6h PI, and increased for both cells studied until 72h PI. The IBDV re-isolation performed by infecting chicken embryos showed positive results at all PI studied. The intracellular IBDV RNA was shown by RT-PCR, where all tested samples were positive. However, the cytopathic effect (CPE) caused by IBDV infection was pronounced at 18h for Vero and 36h for CER cells. The RT-PCR was done to detect IBDV RNA from the bursa samples, where all were positive. Regarding to IBDV isolation, 20% of samples tested were negative in Vero cells, where all were positive in CER cells infection. The findings allow concluding that CER cell can be a new and appropriated tool for IBDV isolation.

INTRODUCTION

Avibirnavirus is the causative agent of Gumboro Disease (GD) an economical and important disease to poultry industry. Infectious bursal disease virus (IBDV) whose genome consists of two segments (A and B) of double-strand RNA, causes an acute, and contagious disease in young chickens according to Betch *et al*. (1988), Kibenge *et al*. (1988) and van der Berg (2000).

Diagnosis of IBDV infections in chickens, eggs or primary cell cultures is extensively employed, although these techniques are not practical as a routine analysis because they are expensive and time consuming (Jackwood & Jackwood, 1994). In addition, the use of CER cells as an alternative for IBDV field samples isolation, cytopathic effects observation, and virus propagation was not well studied. Furthermore, the appearance of IBDV new serotypes with different degrees of pathogenicity, new biological systems including virus isolation and propagation are necessary, even to produce new kinds of vaccines (Di Fabio *et al*., 1999; Eterradossi *et al*., 1999; van der Berg *et al*., 1991; Cardoso *et al*., 2000).

This study was carried out to demonstrate that chicken embryo related cells (CER) could be and appropriated tool to isolate IBDV from field samples.

MATERIAL AND METHODS

Continuous cell line

The Vero cells passage 58 (African green monkey kidney) was obtained

from Adolfo Lutz Institute, São Paulo, Brazil. The chicken embryo related cell (CER) was kindly supplied by Dr. Clarice W. Arns, Department of Microbiology and Immunology, University of Campinas, São Paulo, Brazil. The growth medium for CER and Vero cells was used as described by Cardoso *et al*. (2000) and Ferreira *et al*. (2003).

IBDV isolation and cytopathic effect (CPE) observation

Twenty bursa samples, presenting macroscopic signs of IBDV infection, were collected (with technical assistance of Merial Laboratories). The samples were isolated and after adaptation by 3 consecutive blind passages in Vero and CER cells monolayers, the kinetic study was performed according to Cardoso *et al*. (2000). Briefly, confluent monolayers of both cell cultured in 25 cm^3 flasks were infected with the field strain ($10^{2.7}$ EID_{50}/mL) at a multiplicity of infection of 5. The virus was absorbed at 37°C in a humidified CO_2 incubator for 1 hour. The inoculum was removed and 3 mL of maintenance medium consisting of minimum essential medium (MEM), antibiotics and 2% of calf serum was added as recommended by Cardoso *et al*. (2000). The CPE was graded from + (25%) to ++++ (100%) as described before (Lukert & Davis, 1974). At 6, 12, 18, 24, 36, 48, 60 and 72 h after infection the supernatant was removed and used for extra-cellular IBDV detection by DAS-ELISA and embryos infection (Lukert & Davis, 1974). The RT-PCR and CPE observation were used to detect the cell-associated virus from infected monolayers, either for Vero and CER cells.

Titration of IBDV isolates in specific pathogen free embryonating eggs

Infected monolayers at 6, 12, 18, 24, 36, 48, 60 and 72 h post-infection (PI) were harvest three times and submitted to 10-day-old specific pathogen free embryonating eggs titration and each titre sample (SPF) was calculated by median embryo infectious dose (EID_{50}/mL) method described previously (Reed & Müench, 1938).

Double antibody sandwich ELISA (DAS-ELISA)

The DAS-ELISA was applied to detect extra-cellular IBDV according to Cardoso *et al*. (1998). The specificity was calculated using the non-infected monolayers supernatant and by the use of Newcastle disease virus, reo and influenza A virus detection.

Reverse Transcriptase/Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed to detect viral RNA from infected monolayers and directly from bursa suspension. First, the respective suspension of bursae prepared according to Cardoso *et al*. (1998) was submitted to digestion overnight at 4°C using lysis buffer containing PBS 0.5M NaCl and 20mg/mL proteinase K and after digestion the RNA extraction was performed using Trizol® reagent. Extracted RNA was precipitated using ethanol and dissolved in 90% dimethyl-sulfoxide (DMSO). The precipitated RNA was re-suspended in 100 mL DMSO solution and incubated at 98°C for 5 min. Briefly, 1 mL of RNA in DMSO was 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') as previously described (Tham *et al*., 1995). 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 0.5 mg/mL ethidium bromide. The specificity of RT-PCR was performed using non-infected bursae samples and the RNA extracted from bovine rotavirus.

Specificity of RT-PCR assay

Specificity of RT-PCR was performed by using three other RNA viruses (reovirus S1133, Newcastle disease virus and Rotavirus). The amplicons were visualized in 1.5% agarose gel with 0.5 mg/mL of ethidium bromide.

Histopathology examination of bursae (HE)

The histopathology analysis was performed as described by Cardoso *et al*. (1998). Tissues were fixed in 10% neutral buffered formalin, processed to paraffin and the sections of bursae were cut at 4-5 mm and stained with haematoxylin and eosin. The score used to classified bursal demage followed the standard procedure described by Tanimura *et al*. (1995).

Statistical analysis

The experiment was performed using a completely randomised design schedule and the data (IBDV detected values on tissues - negative control) were submitted to analysis of variance (ANOVA) using the General Linear Model (GLM) of Statistical Analysis System (SAS, 1999). Means were compared by Duncan multiple range test.

RESULTS

The IBDV field strains were isolated in CER and Vero cells and after propagated when the CPE was observed. The CPE was characterized by a market granulation of cell cytoplasm, and gradually detached from the monolayers (Figure 1) at 36 hour PI for CER cell, however it was pronounced in Vero cell since at 18 hour PI (Table 1). The extra-cellular IBDV, titrated by embryonating egg infection, demonstrated that at 60 and 72 hour PI high virus titres obtained from CER infected monolayers (Table 1). The cell associated IBDV showed titre of 2.8 EID_{50}/mL, where no virus could be detected from the supernatant of infected CER and Vero cells. On the other hand, extra-cellular virus had an increase in titre after 36 hour PI detected by DAS-ELISA from the infected Vero and CER supernatant (Table1). The viral RNA from IBDV was confirmed by RT-PCR technique performed on CER and Vero infected monolayers (Figure 2). The IBDV RNA was observed, at all PI when none CPE was observed (Table 2). The CPE involved cell rounding, detachment and lysis (Figure 1). All bursa samples showed severe atrophy of follicles, necrosis and oedema compatible with IBDV infection, and all presented viral RNA of IBDV (Table 2).

Table 1 - Comparison between DAS-ELISA, RT-PCR and embryos infection (EID_{50}/mL) techniques to detect IBDV intracellular and extra cellular particles from infected cells, where no differences were observed between techniques.

PI (h)	Intracellular virus				Extra-cellular virus			
	CER		VERO		CER		VERO	
	RT-PCR	CPE[3]	RT-PCR	CPE	DAS-ELISA[1]	EID[2]	DAS-ELISA	EID
0	_	_	_	_		-	-	-
6	+	_	+	_	0.12	≤2.0	0.13	≤2.0
12	+	_	+	_	0.14	2.6	0.28	2.7
18	+	_	+	+	0.23	2.8	0.32	2.8
24	+	_	+	+	0.25	3.2	0.35	3.2
36	+	++	+	+	0.34	3.9	0.37	3.4
48	+	++	+	+	0.37	4.0	0.43	3.6
60	+	++	+	+	0.45	4.9	0.45	4.6
72	+	+++	+	+	0.49	4.8	0.48	4.8

1 - Positive/negative cut-off= 0.055 (mean±S.D.). 2 - Median embryo infectious dose (log_{10} EID_{50}/mL). 3 - Cytopathic effect graded as described in material and methods section.

Table 2- Comparison between histopathology, RT-PCR and cell isolation to diagnosis the IBDV infection in field samples.

Samples	IBDV detection		IBDV isolation[2]	
	Histopathology[1]	RT-PCR	CER	VERO
S-1	++	+	++++	++++
S-2	+++	+	++++	+
S-3	++++	+	++++	++
S-4	++++	+	++++	+
S-5	+	+	+	+
S-6	++++	+	+++	+++
S-7	++++	+	++++	+++
S-8	++++	+	+	+++
S-9	++	+	++++	+
S-10	+++	+	+	+++
S-11	+	+	++++	+
S-12	++++	+	+	-
S-13	++++	+	+	-
S-14	++++	+	+	-
S-15	++	+	+	-
S-16	+++	+	+	++
S-17	+	+	+++	+++
S-18	++++	+	++++	++++
S-19	++++	+	+	++++
S-20	++++	+	+	++++
Positive	++++	+	++++	++++
Negative		_	-	-

1 - Scores: +, minimum lymphoid necrosis; ++, moderate lymphoid depletion; +++, lymphoid depletion multifocal haemorrage; ++++, severe lymphoid atrophy and necrosis; -, normal. 2 - The cytopathic effect graded from (+)25% to (++++)100%. (-) not detected.

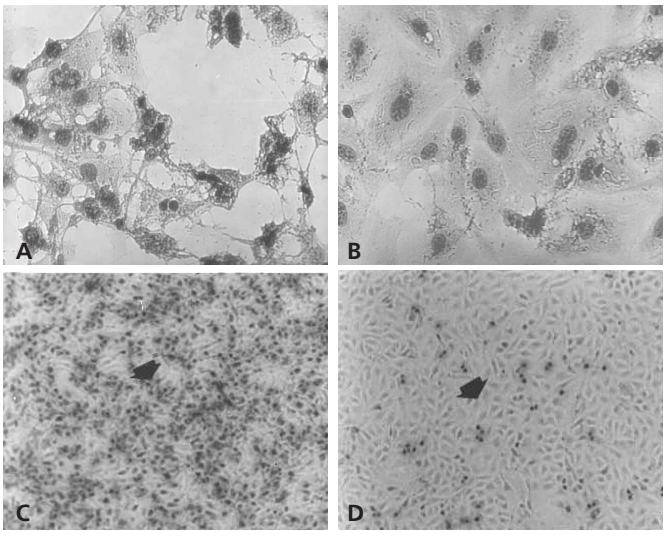

Figure 1 - A) CPE observed in CER cell after IBDV propagation (200X); B) CER uninfected cell; C) CPE observed in Vero cell after IBDV propagation (100X); D) Vero uninfected cell.

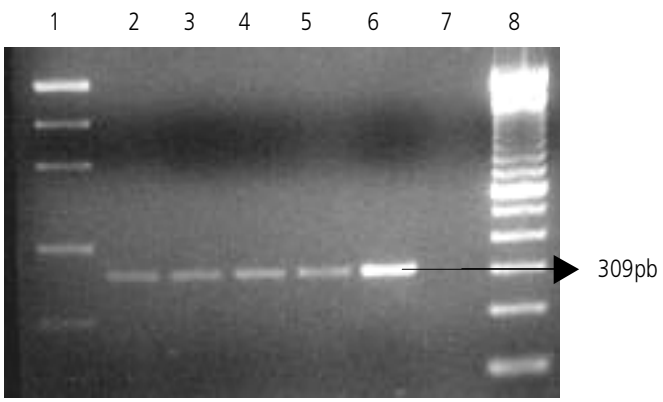

Figure 2 - 1) Low Mass Ladder -5µg; 2-5) Bursa samples; 6) positive control; 7) negative control; 8) 1Kb ladder.

DISCUSSION

The complexity and the nature of IBDV infection require a diversity approach to virus diagnosis. Molecular virology and avian immunology have made considerable progress and should generate new tools in the near future. For instance, the field IBDV isolation should be essential and decisive for virus identification and genetic studies. The mammalian continuous cells lines would more suitable for this purpose, with several advantages over primary cells line. So far, primary avian cell suspensions may contain exogenous avian virus not found in mammalian cell line, which could interfere with a given test or vaccine production (van der Berg, 2000). Nevertheless, this is the first time of field IBDV isolation in CER cells. These cells have been used and the results demonstrated easier to culture than chicken embryo fibroblast (CEF) (Lukert & Davis, 1974) for classical strain propagation (Cardoso *et al.*, 2000). In this study the CPE observed at 18 hour PI from infected Vero monolayers was different from CER infection, when the CPE could be observed at 36 hour PI. The BGM-70 cells (baby monkey kidney cell line) has been used for virus neutralization test also which can be an advantage over CER cells, however neutralization assay should be tested in a near future (Jackwood *et al.*, 1986). Actually, the CPE occurred during the first passage at 72 hour PI, however 2 more blind passages were done to certify IBDV propagation. Thus, the time required for extra-cellular virus detection was almost the same for CER and Vero cell, according to previously studies (Cardoso *et al.*, 1998; Cardoso *et al.*, 2000; Ferreira *et al.*, 2003). The cell associated virus detected by eggs inoculation demonstrated the high virus levels at 60 hour PI and 72 hour PI, showed by CER and Vero monolayers infection. The RT-PCR detected IBDV RNA from infected monolayers and respective supernatant when none CPE was observed. Therefore, in this study it could be possible to confirm in early periods virus replication performing RT-PCR, which can improve faster epidemiological study. The RT-PCR applied in tissue samples positive to IBDV infection by histopathology analysis, showed viral RNA amplification before IBDV isolation (Tham *et al.*, 1995). Finally, the development of safe laboratory techniques that could isolate and propagate IBDV field strains might be considerable. Our results showed the capacity of CER cell to isolate IBDV from all clinical samples and respective propagation, as already demonstrated for classical virus, where 20% was negative for Vero cells system. Nevertheless, this is the first time of field IBDV isolation in CER cells and could be considered useful tool for routine diagnosis of the disease.

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