BJVP
Brazilian Journal of Veterinary Pathology

Original Full Paper

Poult Intestinal Organ Culture for Propagation of Turkey *Coronavirus* and Assessment of Host-virus Interactions

Deriane E. Gomes, Heitor F. Ferrari, Ana C. G. Rosa, Andrea F. Garcia, Lívia C. Bregano, Alexandre L. Andrade, Tereza C. Cardoso

UNESP - São Paulo State University, Laboratory of Virology, DAPSA, Veterinary School, Araçatuba, SP, Brazil
Corresponding author: Tereza C. Cardoso, UNESP - São Paulo State University, Faculdade de Medicina Veterinária, Rua Clovis Pestana 793, Araçatuba, SP, 16050-680, Brazil +55 18 36363200
E-mail: tcardoso@fmva.unesp.br

Submitted January 5th 2011, Accepted February 17th 2011

Abstract

Infection of young poults with turkey coronavirus (TCoV) produces a syndrome characterized by acute enteritis, diarrhea, anorexia, ruffled feathers, decreased body weight gain and uneven flock growth. The objective of this study was to standardize an intestinal organ culture (IOC) in order to assess host-virus interaction related to apoptosis. For this purpose the Brazilian strain (TCoV/Brazil/2006 with GenBank accession number FJ188401), was used for infection. Infected IOC cells had mitochondrial dysfunction and initial nuclear activation with MTT value of 90.7 (± 2.4) and apoptotic factor 2.21 (± 2.1), considered statistically different from uninfected IOC cells ($p > 0.05$). The kinetics of TCoV antigens and viral RNA was directly correlated to annexin-V, caspases- 2 and -3, p53, BCl-2 antigens at 24, 72 and 96 h post-infection (p.i.). Morphological and biochemical features of apoptosis, such as *in situ* nuclear fragmentation (TUNEL and annexin-V) and DNA ladder formation were also detected in infected cells at all assayed p.i. intervals. Moreover, different from other coronaviruses, the expression of both effective caspase-2 and -3 and p53 antigens were considered lower. However, at all p.i., the BCl-2 antigens were expressed quantitatively and qualitatively as viral antigen measured by immunofluorescence microscopy analysis. Because the diagnosis of TCoV infection is only performed by infecting embryonated poult eggs, the pathological characteristics related to host-virus interaction remain unclear. This is the first report on apoptosis of TCoV infected IOC, and reveals that it may be useful immunological method to assess virus pathogenesis.

Key Words: Apoptosis, *Turkey coronavirus* (TCoV); intestinal organ culture.

Introduction

In the year 2006, an outbreak of Poult Enteritis Mortality Syndrome (PEMS) was detected for the first time caused by a group 3 *Coronavirus* [14] in Brazil. Since then, the respective virus was isolated and partially sequenced [4].

Previous works have demonstrated that TCoV is able to grow in poult embryos inoculated via the amniotic route at 23-25 days of incubation [3, 9]. Many efforts have been made to establish a cell culture able to support TCoV isolation and further propagation [3] but none have been successful. However, the use of intestinal organ cultures seems to be an alternative for *in vitro* studies reported before [9]. The greatest advantage of using cells cultures instead of embryos for inoculation is the possibility of *in vitro* studies related to the natural environment of virus infection, with no interference from other organs or biochemical cell signaling. Many studies on other coronaviruses have demonstrated the *in vitro* assessment of apoptotic pathways by infecting a variety of cell cultures [6, 7, 8, 11, 13]. However, due to the obstacles related to growing TCoV in a trustworthy cell culture system, there has been no information of the apoptotic mechanism for this virus until now.

To enhance cell viability and facilitate replication, viruses possess multiple mechanisms to inhibit the host response [2]. Apoptosis serves as an innate cellular response to infection that limits both the

time and intracellular viral replication. Thus, the present investigation was conducted to elucidate some host-virus interactions using the TCoV isolated in Brazil in poult intestinal cell culture. The TUNEL assay and indirect immunofluorescence assays for the expression of pro- and anti-apoptotic antigens (p53, annexin-V and BCl-2) and caspase-2 and -3 antigens were performed to measure the effects of infection at 24, 72 and 96 h post-infection (p.i.). Viral antigens and RNA were also considered for analysis.

Material and Methods

Virus

The TCoV strain (TCoV/Brazil/2006 accession number FJ188401) used in the present investigation was isolated from field cases of PEMS in 2007 [14]. The virus was propagated in our laboratory using 24-day-old embryonated commercial poults eggs, inoculated via the amniotic sac route. The intestines of inoculated embryos were collected 48 h after inoculation, homogenized, and clarified by centrifugation at 3000 x *g* for 30 min. The supernatants were filtered through 0.45 µm and 0.25 µm syringe filters (Corning®, Life Sciences, Axygen, Inc. Union City, CA), respectively and stored until used. The suspension was tested for other avian pathogens by molecular tools and for *Mycoplasm* contamination using commercial Kit (Sigma-Aldrich®).

Intestinal organ culture (IOC)

The intestinal organ culture procedure was modified from the previously published method [9, 12]. A 0.5 cm long segment of the ileum between the caeca was collected. All segments were collected under sterile conditions, opened longitudinally, and immediately washed twice with calcium- and magnesium-free Hanks balanced salt solution (HBSS, Sigma-Aldrich®, St. Louis, MO). The segments were from healthy 1-day poults, negative for TCoV, reovirus, rotavirus, and turkey astrovirus by molecular search. The fragments were placed in six-well plates with non-enzymatic dissociation buffer (pH 7.2) containing 1 mM EDTA, 10 ng/ml of EGF (epidermal growth factor, Sigma-Aldrich®), 0.5 mM dithiothreitol and 10% antibiotic solution (Sigma-Aldrich®), and allowed to incubate with shaking at 2, 000 x *g* at room temperature. The cell aggregates were placed in DMEM/Hams Nutrient F12 [Dulbecco´s Modified Eagle´s Medium/ Hams Nutrient F12 (1:1); Gibco-BRL, Grand island, NY, USA] plus 10% of an antibiotic solution, 1 mM glutamine and 10% fetal bovine serum (FBS). The cultures were incubated at 37° C in a 5% CO_2 atmosphere and the medium was replaced every two days.

Experimental inoculation

In order to perform TCoV infection, aliquots of $10^{2.3}$ EID (embryo infectious dose)/ml stored in 1 mL vials and kept under -86°C were used [4]. After removal of the medium, the cultures were covered by 1 ml of the inoculum and left one hour at 37ºC to facilitate virus adsorption. The cultures were washed five times with sterile PBS (phosphate buffered saline), pH 7.4, and new media was added. Then, the infected cultures and uninfected cells were observed twice a day at 24 h, 72 h and 96 h post-infection (p.i.), after which the supernatant was removed and the following steps were carried out on the infected cultures.

Extraction of RNA and reverse transcription-polymerase chain reaction (RT-PCR)

Viral RNA was extracted from each culture at the previously stated post-infection times using the Pure Link® Viral RNA/DNA kit (Invitrogen™, Carlsbad, CA), following the manufacturer's instructions. The reverse transcription - polymerase chain reaction (RT-PCR) was performed as described previously (Gomes et al., 2010). A portion of the S2 gene was amplified using 10 pmol of oligonucleotides: S2 sense (1178 position in the genome) forward and S2 anti-sense (2073 position in the genome) reverse, both designed for this study. The expected products (890 bp) were analyzed by 1.5% agarose gel electrophoresis and visualized by staining with ethidium bromide and UV illumination. The viral antigens were detected using immunofluorescent assay, as described previously [5].

Analysis of apoptosis

MTT assay and Apoptosis ELISA

The *TOXI- 1* assay was performed to evaluate the proliferative rate of viable cells according to the manufacturer's instructions (Sigma-Aldrich®). The assay is based on the mitochondria metabolization of the 3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyl tetrazolium bromide (MTT). Briefly, the IOC cultures were placed in each of a 6-well-plate with 1 ml of medium per well, and after 200 µl of MTT solution was added at a concentration of 5mg/ml, followed 4h to allow the MTT to be metabolized. The medium was then removed and cells were ressuspended in formazon in 2ml DMSO. The OD_{560} was read, and was found to be directly correlated with cell quantity, as expected.

Apoptotic ELISA was used in this study according to the instructions provided by manufacturer (Genentech®, South San Francisco, CA). Briefly, 100µl of the IOC individual lysates were placed into a streptavidin-coated microtiter plate (5µg/well) and a mixture of anti-histone (1:100) and anti-DNA (1:50) antibodies was added. During incubation, the anti-histone antibody binds to histone components of the nucleossomes and simultaneously the immunocomplexes is captured by streptavidin via biotinylation. Additionally, the anti-DNA peroxidase reacts with the DNA components of the nucleossomes. Color was developed by addition of ABTS substrate (Sigma-Aldrich®), and the OD_{405} was measured in a Multiskan ELISA reader (Thermolab®, South San Francisco, CA) . The apoptotic factor was calculated by

the formula: absorbance of the sample (dying/dead cells)/absorbance of the negative control.

Indirect immunofluorescence assay to search annexin-V, caspases-2 and -3 and BCl-2 antigens

The intestinal cultures were washed three times in PBS and fixed in 4% paraformaldehyde for 24 h at 4°C. Samples were then rinsed with PBS and permeabilized with proteinase *K* (10 µg /mL) for 15 min at room temperature. The details of all antibodies used in this study are described in Table 1. After pre-treatment, the cells were incubated overnight with the primary antibodies (mouse anti-annexin-V, p53, caspase-2, -3 and Bcl-2) diluted in Antibody diluent DAKO (S3022, Dako North America®, Inc., Carpinteria, CA). The cells were then incubated for 24 h at 4°C with the secondary antibody (StreptAvidin®-Cy3, Sigma-Aldrich®). Omission of the primary antibody was used as a negative control for the different antibodies. Subsequently, all samples were counterstained with 1 mg/ml of 4`,6`-diamidino-2-phenylindole hydrochloride (DAPI) for 15 min at room temperature before mounting the slides in the dark.

Table 1: Details of the primary antibodies used

	Antibody	Dilution	Species	Supplier
annexin-V	Monoclonal anti-annexin-V	1:4000	Mouse	Sigma-Aldrich® (cat # A8604)
caspase-2	Monoclonal anti-caspase-2 (ICH1 L)	1:400	Rabbit	Sigma-Aldrich® (cat # C7349)
caspase-3	Monoclonal anti-caspase-3, Active	1:1000	Rabbit	Sigma-Aldrich® (cat # C8487)
Bcl-2	Monoclonal anti-Bcl-2	1:500	Mouse	Sigma-Aldrich® (cat # B9804)
p53	Monoclonal anti-p53	1:100	Mouse	Sigma-Aldrich® (cat # P3737)

TUNEL assay

TUNEL staining was performed following the manufacturer's instructions (A35126 APO-BrdU™ TUNEL Assay Kit, Invitrogen™). Briefly, IOC were washed twice in wash buffer (ABO-BRDU Kit) and incubated at 4°C for 12 h in prepared DNA-labeling solution (ABO-BRDU Kit) containing TdT enzyme, BrdUTP, TdT reaction buffer and distilled water. After washing twice in rinse buffer (ABO-BRDU Kit), the sections were incubated in the dark for 30 min at room temperature with the antibody solution (fluorescein-labeled anti-BrdU monoclonal antibody and Rinse buffer). The positive control consisted of slides pre-incubated with 3U/ml of DNAse for 1 h at 37°C. A negative control was incubated without the TdT enzyme.

Semi quantification and data analysis

The levels of antigens detected in the TUNEL, p53, annexin-V, caspases-2 and -3, and BCl-2 assays were semi-quantified according to the intensity of the immunofluorescence reactions at 72 h after infection and calculated as number of positive cells/mm^2. IOC slides were examined on two separate occasions by two observers without prior knowledge of classification. The images were collected under an Axio Imager A.1 light microscope connected to AxioCam MRc (Carl Zeiss Oberkochen, Germany), and micrographs were processed with Axiovision 4.7 software (Carl Zeiss). All data presented were obtained from at least three independent experiments and are presented as mean ± standard deviation (± s.d). Statistical significance was assessed by Student´s *t*-test, carried out using ANOVA software.

Results

Morphology of infected and uninfected cells IOC cells

The morphology of IOC cells is illustrated in Fig. 1A and B. The Fig. 2 shows the percentage of reactive IOC cells to apoptotic markers and TCoV antigens at different p.i periods and antigens labeling are illustrated in Fig. 3 (A, B, C and D). BCl-2 antigen was detected in > 80% of the infected IOC cells at all p.i. periods (Fig. 3A). At 24 h p.i. 40 % of the cells

were positive for TUNEL assay and annexin-V (Fig. 3B and C). In contrast, caspase-2 and 3 and p53 antigens were lower in all p.i periods (Fig. 3D). The kinetic of TCoV antigens detection revealed > 20 % positive cells at 24 h p.i. and an increase of this phenomenon in the last periods (Fig. 2).

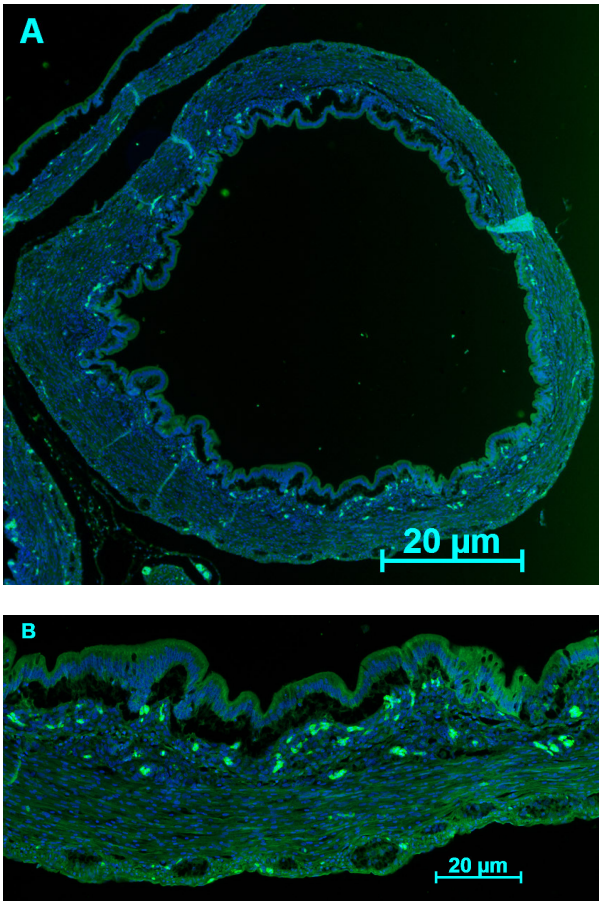

Figure 1. Morphology of intestinal organ culture (IOC). The IOC cells plated on 2% matrigel-coated 6-well plates are shown in panel (A) sagital plane and (B) longitudinal plane. IF, 40x.

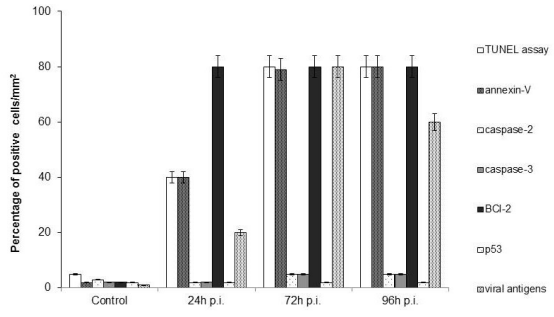

Figure 2. Relationship between annexin-V, caspase-2 and -3, BCl-2 and p53 activation and TCoV antigens after infection at 24 h, 72 h and 96h p.i. The results are expressed as the percentage of positive cells/mm^2.

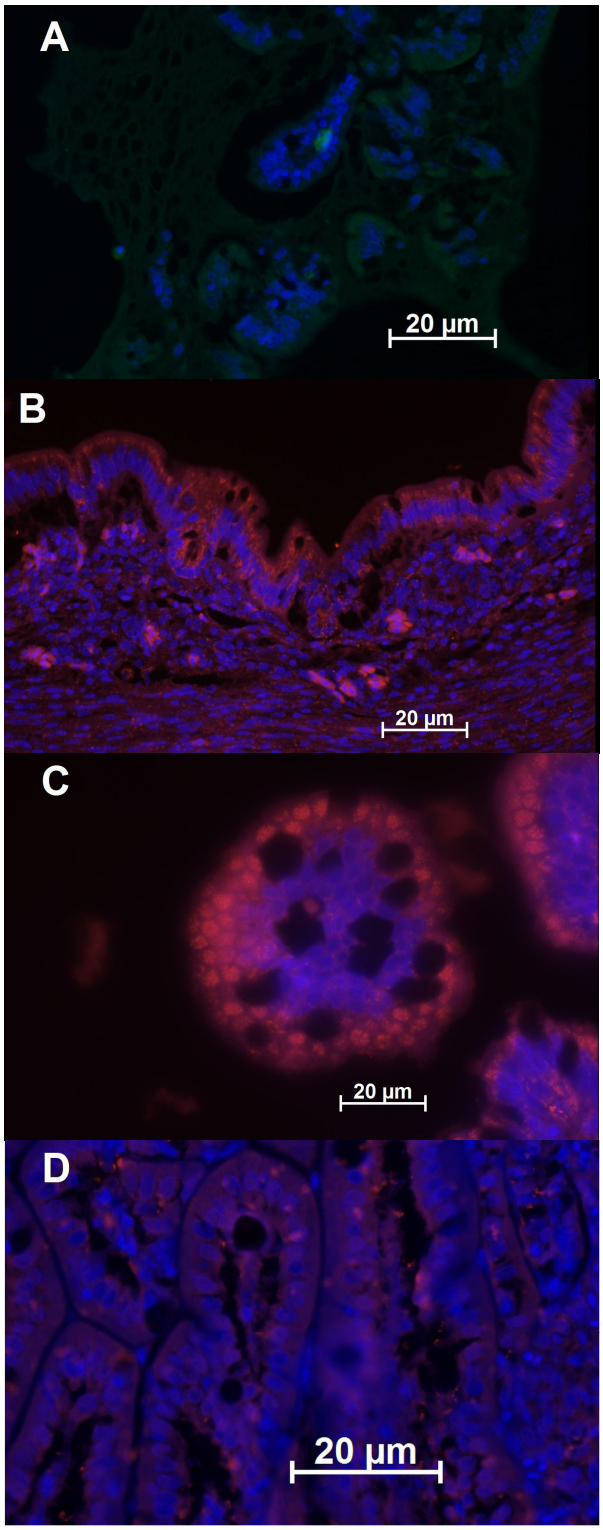

Figure 3. Representative picture of BCl-2 (A), TUNEL assay (B), annexin-V (C) and p53 antigens expressed in IOC infected with TCoV at 24h p.i. labeled by Cy3 dye and observed under fluorescent microscopy. IF, 40x.

MTT and apoptotic ELISA results

The results of cell proliferation measured by MTT and apoptotic rates are expressed on Table 2. After 72 h post-infection, both cell proliferation 90.7 (± 2.4) and apoptotic factor 2.21 (± 2.1) were considered

statistically different from uninfected IOC cells ($p > 0.05$). These results revealed that TCoV infection alters the mitochondrial functions and both histones and host DNA were released from nucleus indicating early apoptotic stage. In contrast, the uninfected IOC did not show any statistical differences among post-infection periods, MTT and apoptotic ELISA results.

Table 2: Results obtained from MTT and apoptotic ELISA assays in uninfected IOC and TCoV-infected IOC

	TOXI 1[c] (% viability)			Apoptotic ELISA[d]		
Post-infection (p.i)	**24h**	**72h**	**96h**	**24h**	**72h**	**96h**
Uninfected IOC	100	100	95	1[a]	1[a]	1[a]
TCoV-infected IOC	100	90.7 (± 2.4)	83.5 (± 2.9)	9.05[b] (± 2.3)	2.21[b] (± 2.1)	2.0[b] (± 0.81)

Within a column, values with different superscripts differ statistically (a and b, $p \leq 0.05$). [c] *TOXI 1* assay results are expressed as a percentage normalized against uninfected IOC ($p > 0.05$). [d]The rate of apoptotic ELISA is shown as the apoptotic factor.

Discussion

Apoptosis represents an important antivirus defense mechanism in host cells, and viruses have evolved strategies to counteract and regulate apoptosis in order to maximize the production of virus progeny and promote viral spread to neighboring cells. In spite of the fact that turkey *Coronavirus* enteritis was identified in Minnesota between 1951 and 1971, in Great Britain in 2006 and, recently, in Brazil in 2007 [1, 10, 14] this report represents the first investigation into whether or not TCoV could induce apoptosis when propagated in intestinal organ culture. Moreover the IOC cells system was established and after virus infection the cells demonstrated more citotoxic effects than non-infected ones.

This study characterized certain aspects related to apoptotic pathways. Before 24 h p.i., neither morphological changes nor protein expression could be detected, in spite of the presence of viral RNA, which was identified by RT-PCR at all p.i. periods. It is known that TCoV and IBV (infectious bronchitis virus) are closely related, and it is also known that IBV corresponded to a prototype virus of the *Coronaviridae* family. Since IBV-infected Vero cells showed syncytium formation followed by caspase-dependent apoptosis [8], TCoV in this study seems not to have activated the same effecter pathway; since fluorescent signals observed among infected cells at all p.i. periods were considered the lowest. It has also been demonstrated that IBV infected-Vero cells began to show positive for the TUNEL-signal at 24 h p.i. [8]. In addition, the positive TUNEL signals were found mainly in cells that formed syncytia, indicating that apoptosis was tightly associated with productive virus replication. By analysis of results presented here, the same phenomenon could be described; TCoV productive virus replication was intense at 72 h p.i., whereas TUNEL and annexin-V-positive signals could be visualized in 80% of the infected intestinal cells. Therefore, it is conceivable that TCoV has genetically evolved into a group 3 *Coronavirus* [6], but has some differences in function from the *Coronaviridae* family, especially regarding the process of apoptosis, as demonstrated in the present investigation. As a result of the complex interplay between virus and host, infection of different cell types by the same virus does not necessary activate the same cell death pathway [6, 8, 11, 13].

Another study has showed that IBV infection does not affect the expression of p53 protein in host cells [6]. This finding is in accordance with results reported in the present investigation, where p53 antigens could not be observed in both TCoV-uninfected and infected intestinal cells. In some coronaviruses, both initiator caspases are activated during the course of apoptosis [13]. The Fas-signaling pathway, in which both initiator caspases are activated via cleavage and translocation of Bid, has been suggested as the mechanism of apoptosis [2]. Taken together with our results, it is possible to infer that TCoV infection indirectly activates cytochrome c release, which induces a high expression of BCl-2 antigens among infected intestinal cells. However, BCl-2, TUNEL and annexin-V expression did not affect the susceptibility of infection, the kinetics or viral replication; this implies that apoptosis is involved in facilitating TCoV infection in IOC cultures. Moreover, TCoV infection seems to affect also mitochondrial function of the infected cells as revealed by MTT assay in this study. However, the pathway of this activation must be addressed in future studies.

Given that analysis of poult host-virus interactions has suggested that there are significant differences in apoptotic mechanisms of TCoV infection, it is extremely necessary to carry out future investigations using other cell lines that can support TCoV infection to elucidate such mechanisms.

Conclusions

Our study brings new insights into the role of TCoV infection in an *in vitro* biological system. Therefore, the only biological model to propagate TCoV is the inoculation in embryonated eggs and no study was made to improve the host virus interaction until now. These achieves offer the opportunity to use the IOC system as a model in the study of many mechanisms of virus survival.

Conflict of interest statement

None declared.

Acknowledgements

This research was supported by FAPESP (05/52994-3; 07/56041-6; 08/50380-6) and CNPq/47226/07-0. TC Cardoso is a fellowship of CNPq.

References

1. ADAMS, NR., HOFSTAD, HS. Isolation of transmissible enteritis agent of turkeys in avian embryos. *Avian Dis*., 1971, 15, 426-433.
2. BORDI, L., CASTILLETTI, C., FALSCA, L., CICCOSANTI, F., CALCATERRA, S., ROZERA, G., DI CARO, A., ZANIRATTI, S., RINALDI, A., IPPOLITO, G., PIACENTINI, M., CAPOBIANCHI, MR. Bcl-2 inhibits the caspase-dependent apoptosis induced by SARS-CoV without affecting virus replication kinetics. *Arch. Virol*., 2006, 151, 369-377.
3. BROWN, TP., GARCIA, AP., KELLY, L. Spiking mortality of turkey poults: I. Experimental reproduction in isolation facilities. *Avian Dis*., 1997, 41, 604-609.
4. CARDOSO, TC., CASTANHEIRA, TL., TEIXEIRA, MCB, ROSA, AC., HIRATA, KY., ASTOLPHI, RD., LUVIZOTTO, MC. Validation of an immunohistochemistry assay to detect turkey coronavirus: a rapid and simple screening tool for limited resource settings. *Poult Sci*., 2008, 87, 1347-1352.
5. GOMES, DE., HIRATA, KS., SAHEKI, K., ROSA, ACG., LUVIZOTTO, MCR., CARDOSO, TC. Pathology and virus tissue distribution of turkey *Coronavirus* (TCoV) in experimentally infected chicks and turkey poults. *J. Comp. Pathol*., 2010, 143, 8-13.
6. LI, FQ., TAM, JP., LIU, DX. Cell cycle arrest and apoptosis induced by the coronavirus infectious bronchitis virus in the absence of p53. *Virology*., 2007, 365, 435-445.
7. LIN, TL., LOA, CC., TSAI, SC., WU, CC., BRYAN, TA., THACKER, HL., HOOPER, T., SCHRADER, D. Characterization of turkey coronavirus from turkey poults with acute enteritis. *Vet. Microbiol*., 2002, 84, 179-186.
8. LIU, C., XU, HY., LIU, DX. Induction of caspase-dependent apoptosis in cultured cells by the avian coronavirus infectious bronchitis virus. *J. Virol*., 2001, 75, 6402-09.
9. LOA, CC., LIN, TL., WU, CC., BRYAN, T., HOOPER, T., SCHRADER, D. Specific mucosal IgA immunity in turkey poults infected with turkey coronavirus. *Vet. Immunol. Immunopathol*., 2001 88, 57-64.
10. NAGARAJA, KV., POMEROY, BS. Secretory antibodies against turkey coronaviral enteritis. *Am. J. Vet. Res*., 1998, 39, 1463-1465.
11. RUGGIERI, A., DI TRANI, L., GATTO, I., VIGNOLO, E., BEDINI, B., ELIA, G., BUONAVOGLIA, C. Canine coronavirus induces apoptosis in cultured cells. *Vet. Microbiol*., 2007, 121:64-72.
12. STAMM, I., MOHR, M., BRIDGER, PS., SCHRÖPFER, E., KÖNIG, M., STOFFREGEN, WC., DEAN-NYSTROM, EA., BALJER, G., MENGE, C. Epithelial and mesenchymal cells in the bovine colonic mucosa differ in their responsiveness to Escherichia coli shiga toxin 1. *Infec. Immunity*., 2008, 76, 5381-5391.
13. SUSUKI, K., MATSUI, Y., MIURA, Y., SENTSUI, H. Equine coronavirus induces apoptosis in cultured cells. *Vet. Microbiol*., 2008, 129, 390-395.
14. TEIXEIRA, MC., LUVIZOTTO, MC., FERRARI, HF., MENDES, AR., DA SILVA, SE., CARDOSO, TC. Detection of turkey coronavirus in commercial turkey poults in Brazil. *Avian Pathol*., 2007, 36, 29-33.

