Malays. Appl. Biol. (2012) 41(1): 47–50

RESEARCH NOTE

REPLICATION OF VERY VIRULENT INFECTIOUS BURSAL DISEASE VIRUS IN PRIMARY CELLS OF THE CHICK EMBRYO CHORIOALLANTOIC MEMBRANE

Mohammed, M.H.*, Hair-Bejo, M., Omar, A.R. and Aini, I.

Faculty of Veterinary Medicine, Universiti Putra Malaysia
43400 UPM Serdang, Selangor, Malaysia
**Email: majed_mohammed@putra.upm.edu.my*

Infectious bursal disease (IBD), an immunosuppressive viral disease, causes significant losses to the poultry industry either by causing high mortality in an acute disease or as a consequence of immunosuppresion (van den Berg, 2000). The IBD virus (IBDV) can infect and grow on various primary cell cultures of avian origin and certain cell lines of mammalian origin. Tissue culture commonly used to propagate IBDV is chicken embryo fibroblast (Lukert and Davis, 1974) or chicken embryo kidney or baby hamster kidney (El-Ebriary *et al.,* 1997) or ovine kidney (Kibenge and MuKenna, 1992) or normal chicken lymphocytes, B-cell lymphoblastoid or rabbit kidney (Rinaldi *et al.,* 1972), baby grivet monkey kidney and M4-104 cells (Jackwood *et al*., 1987). Vero cells are fibroblast like cells fromthe kidney of a normal adult African green monkey (Peilin *et al.,* 1997). Isolation and propagation of very virulent infectious bursal disease virus (vvIBDV) from field strain in primary or secondary cell cultures of chicken embryo origin were found to be very difficult, this poor adaptation of vvIBDV in cell cultures may due to the strain differences in field viruses (Mannan *et al.,* 2009). Recently, a long-term cell culture embryo chorioallantoic membrane (CAM) cells developed from specific pathogenic free (SPF) eggs has been established in our laboratory. Its ability to support replication of the vvIBDV was examined in this study.

Malaysian isolate namely UPM0081 which is characterized as vvIBDV (GenBank accession number : AY520910) (Tan *et al*., 2004) was used in the present study.

The isolate was passaged in specific-pathogen-free embryonated chick-eggs via CAM for 3 times, prior to adaptation in the CAM cells. Primary chick embryo CAM cells were prepared from 9- to 10-day-old specific pathogenic free (SPF) chick embryo. The CAM cells were collected, washed with PBS and digested with trypsin/EDTA. The reaction was stopped by adding DMEM complete growth medium (GIBCO Laboratories, USA) supplemented with 2.0 g NaHCO3, 10% fetal calf serum (FCS), antibiotic of 100 IU/ml penicillin and 100 IU/ml streptomycin. After centrifugation at 1000 g for 10 min, the CAM cells were resuspended in the same medium and filtered through sterile gauze. The CAM cells in the filtrate were distributed on plastic tissue culture flasks and incubated at 37ºC with 5% CO_2.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) was performed in this study to detect viral RNA from infected CAM monolayer cells to confirm virus replication in these cells. Cell culture supernatant and infected monolayers were used for viral RNA extraction using Trizol reagent (GIBCO/BRL Life Technologies) following the method recommended by the manufacturer. RT-PCR was performed by denaturant viral dsRNA using 90% dimethyl sulfoxide, heated at 95ºC for 5 min and cooled on ice for at least 2 min. The reverse transcriptase reaction to synthesize cDNA contained 0.5µl of AMV reaction buffer, 4µl of 10 mmol/l dNTPs, 4µl of 25 mmol/l $MgCl_2$, 20 U of RNase, 0.5µl of AMV reverse transcriptase (*Promega*) and dH_2O were added to a final volume of 20µl. The PCR reaction mixture to amplify the cDNA contained 1 µl of each forward primer p1 (5'-TCA CCG TCC TCA GCT TAC-3', nt 587-604) and reverse primer P2 (5'-TCA GGA TTT GGG ATC AGC-3', nt 1212-1229) (Liu *et al*., 1994) were used to amplify a 643 bp region of VP2 gene located on segment A of IBDV genome. Five µl of cDNA was mixed with 6 µl of *Taq* amplification buffer, 1µl of 10mmol/l dNTPs, 4µl of 25 mmol/l $MgCl_2$, 2.5 U of *Taq* DNA polymerase (*Promega*) and distilled water to a final volume of 50µl. After initial denaturation at 95ºC for 1 min, amplification proceeded in a DNA thermal cycler for 35 cycles of denaturation (94ºC

* To whom correspondence should be addressed.

for 1min), primer annealing (48ºC for 1min) and primer extension (72ºC for 2min), with a final extension at 72ºC for 10 min.

The indirect immunoperoxidase test (IIP) was performed from the first passage. Samples taken from cell cultures were fixed with cold methanol: acetone (50:50 v/v) for 5 min. The glass slides were then immersed in 1% hydrogen peroxide (H_2O_2) in absolute methanol for 30 min. The PBS was then added to the glass slide for 15 min. The glass slides were then air dried. The hyper immune serum (Charles River Laboratories, USA) was diluted 1:1000 with PBS and added to the glass slide incubated for 1 hour at room temperature. The glass slides were then washed 3 times with PBS for 5 min each. The rabbit anti-chicken IgG-HRP conjugated secondary antibody (Bio-Rad, USA) was then added to the glass slides (1: 1000) and incubated for 1 hour at room temperature. DAB substrate solution (DAB reagent set, Invitrogen, USA) was then added to the glass slides and incubated for 10 min in a dark room. The slides were mounted with 50% glycerol in phosphate buffer saline (PBS) and examined under light microscope (Guvenc *et al*., 2004).

To determine impact of cell passages on vvIBDV replication titers, tissue culture effective dose 50 ($TCID_{50}$) was conducted in the CAM cells (Reed and Muench (1938). Ten-fold serial dilution of vvIBDV was prepared in PBS from 10^{-1} to 10^{-10}. A 96 well tissue culture microtitration plate (Titertek, UK) was used to prepare CAM cells monolayers. A 100 µl of each virus dilution was added in each well of first row leaving last two wells as negative control. The plate was incubated at 37ºC for 1 hour to allow adsorption. Then 100 µl of prewarmed maintenance medium was added in each well and again incubated at 37ºC in 5% CO_2. The plate was observed twice daily for CPEs. The highest dilution of virus showing 50% CPEs was considered as end point to calculate $TCID_{50}$.

The vvIBDV strain (UPM0081) was successfully adapted in CAM cells with the formation of CPEs. The CPEs were observed in first passage after five days post inoculation (5dpi) (Fig. 1) while in passage 2 and 3 complete CPEs were recorded at 3 days post inoculation, respectively. The CPE in CAM cells were characterized by aggregates of tiny round refractive cells that later spread to the entire cell sheet. These altered cells eventually detached from the surface, leaving empty areas in the cell culture.

The replication of vvIBDV in infected CAM cells was confirmed by two independent tests; IIPS and RT-PCR. For IIPS, the infected cells showed positive by presence of specific intracytoplasmic brownish coloration (Fig. 2). The results of the RT-PCR assay revealed an amplified fragment from the supernatant of CAM monolayers of the vvIBDV VP2 gene (Fig. 3). There is no unspecific amplification observed in the RNA extracted from uninfected CAM cells.

Many studies showed that vvIBDV isolated from field sample was not able to propagate in cell culture originally (Mannan *et al.,* 2009). Adaptation needs several blind passages in cell culture or embryonated eggs (Yamaguchi *et al.,* 1996). This study was initiated to find an alternative to chick embryo fibroblast cells that could be useful for propagation of vvIBDV from the first time. Normal and confluent monolayer of CAM cells was formed following 24 hours of growth in DMEM growth medium.

We have demonstrated vvIBDV replication in CAM cells, CPE being observed at day 5 post inoculation. The CPE involved cell rounding and some infected cell death from the tissue culture flask

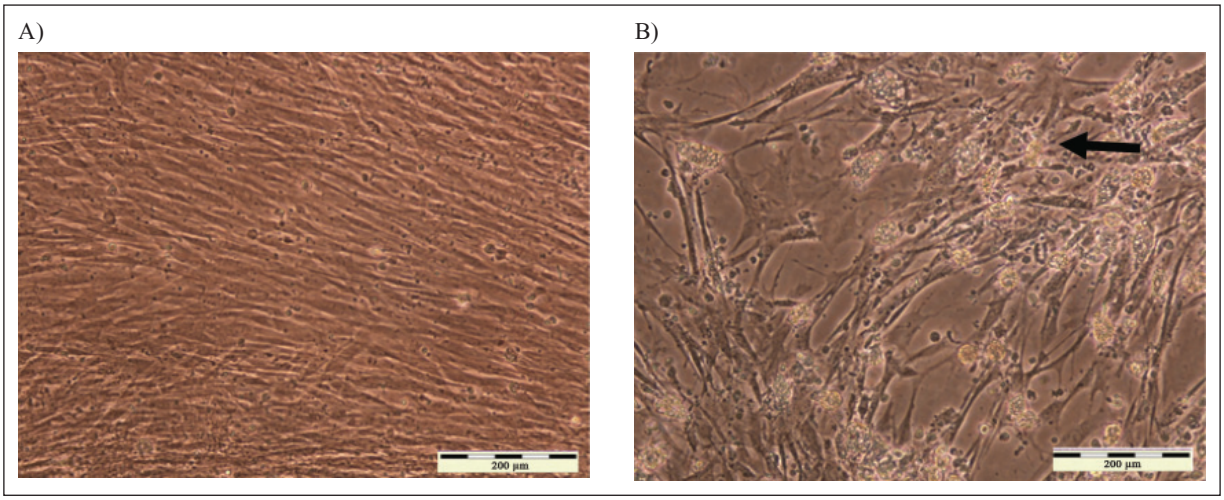

Fig. 1. (A) Uninfected control CAM cells monolayer. (B) Cytopathic effect of UPM0081 isolate of the 1st passage at day 6 pi. The arrow shows cell rounding and clumping. 10x. Bar = 200µm

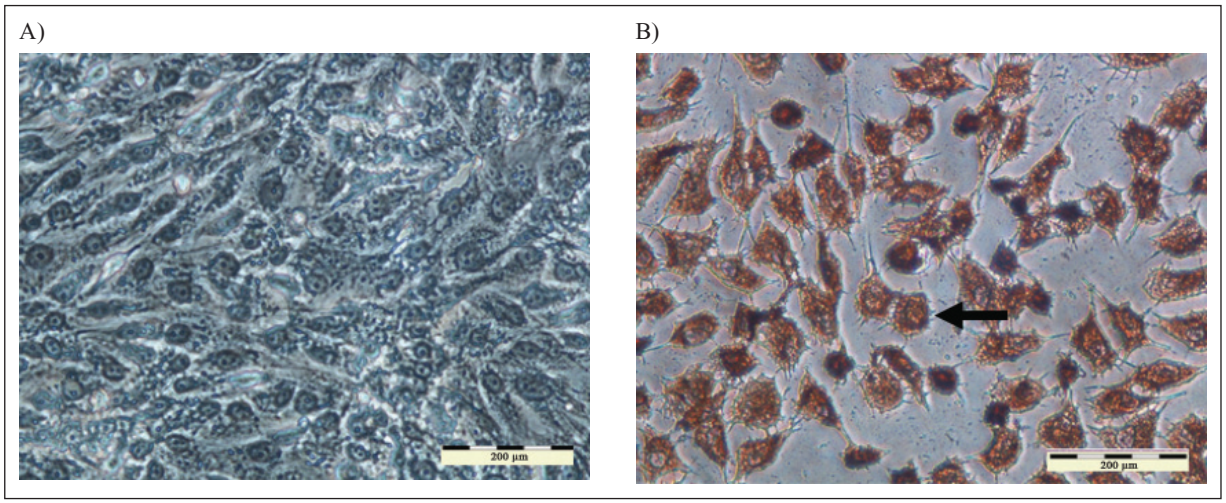

Fig. 2. Identification of vvIBDV in CAM cells culture using infected cell cultures stained with HRP-conjugated antibody. (A) Uninfected control CAM. (B) CAM infected with UPM0081 at 1st passage at day 5 pi. The arrow shows the presence of specific intracytoplasmic brownish coloration. 10x. Bar = 200µm

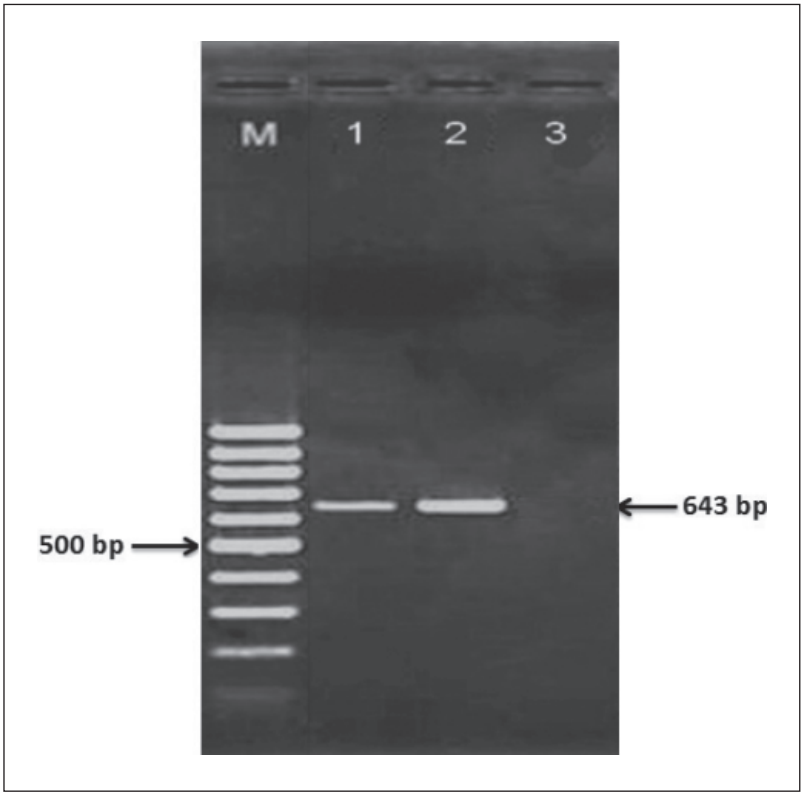

Fig. 3. Agarose gel electrophoresis of amplicons following RT-PCR of vvIBDV RNA extracted from CAM cells: at 6 dpi passage 1 (lane 1) and 3 dpi passage 2 (lane 2); 100 bp DNA ladder markers (lane M).

and float in the medium (Jackwood *et al.,* 1987). vvIBDV antigen was directly detected in the infected CAM cells by using indirect immuno-peroxidase and the viral antigen was observed as brown granular precipitates at the site of antigen localization in the cytoplasm (Guvenc *et al*., 2004).

In this study, the infected monolayers CAM cells (2nd passage) was titrated by $TCID_{50}$. The virus titer was found to be $10^{6.6}$ after 72 hours of infection. Lukert *et al.* (1974) also reported that Vero cells gave titer of approximately 10^5 $TCID_{50}$ after 72 hours post infection coinciding with the

appearance of CPE. Similar finding was reported by Kibenge *et al.* (1988) in Vero cells where they observed the growth pattern of five strains of serotype 1,2 and variant strains of IBDV in these cells. They found titers ranged from 6.85 to 8.35 $\log_{10}$ $TCID_{50}$/ml in Vero cell after 48 hours of infection. With all these studies and finding, we reached to a conclusion that vvIBDV isolated locally can be adapted on CAM cells and gives a good titer from the second passage.

The RT-PCR applied in this study detected IBDV RNA from infected monolayers and respective supernatant of CAM cells using specific primer set of hypervariable region of VP2 gene resulting in generation of a targeted amplicon of 643 bp. This revealed the presence of RNA virus in infected CAM monolayers and confirmed the IBDV replication performing RT-PCR, which can prove the capacity of CAM cells for the propagation of vvIBDV virus.

Finally, the development of safe and reliable laboratory techniques to isolate and propagate vvIBDV field strain in CAM cells for the first time may open a new opportunity to use this cell culture as a tool for routine diagnosis or vaccine production in the future.

ACKNOWLEDGMENT

This project was supported by RUGS, Universiti Putra Malaysia (Grant No 91091).

REFERENCES

El-Ebriary, E.A., EL-Mahidy, S.S. & Khodeir, M.H. 1997. Trials of using cell cultures of evaluation of infectious bursal disease virus vaccines in Egypt. *Assiut Veterinary Medical Journal,* **36**: 105-123.

Guvenc, T., Haziroglu, R., Yarim, M. & Tunca, R. 2004. Diagnosis of infectious bursal disease by immunoperoxidase technique. *Vet Fak Derg*., **51**: 237-238.

Jackwood, D.H., Saif, Y.M. & Hughes, J.H. 1987. Replication of infectious bursal disease virus in continous cell line. *Avian Disease*, **31**: 370-375.

Kibenge, F.S.B., Dhillon, A.S. & Russell, R.G. 1988. Growth of serotype-I and II and variant strains of infectious bursal disease virus in Vero cells. *Avian Disease*, **32**: 298-303.

Kibenge, F.S. & Mukenna, P.K. 1992. Isolation and propagation of infectious bursal disease virus using the ovine kidney continous cell line. *Avian Disease*, **36**: 256-61.

Liu, H., Giambrone, J.J. & Dormitorio, T. 1994. Detection of genetic variations in serotype 1 isolates of infectious bursal disease virus using polymerase chain reaction and restriction endonuclease analysis. *Journal Virolology Methods*, **48**: 281-291.

Lukert, P.D. & Davis, R.B. 1974. Infectious bursal disease virus: Growth and characterization in cell cultures. *Avian Disease*, **18**: 721-738.

Mannan, S., Ibrahim, M., Malik, S.A. & Khwaja, K.N. 2009. Comparative susceptibility of different cell cultures and chicken embryo organ cultures to infectious bursal disease virus of poultry. *Recent Research in Science and Technology,* **1**(6): 294-298.

Peilin, W., Baoxiang, C. & Jinsong, L. 1997. Adaptation and propagation of infectious bursal disease in Vero cells. *Journal of Veterinary Science and Technology,* **27:** 23-24.

Reed, L.J. & Muench, H. 1938. A simple method of estimating fifty percent endpoints. *American Journal of Hygiene*, **27:** 493-497.

Rinaldi, A., Lodetti, E., Cessi, D., Lodirini, E., Cercio, G. & Nardelli, L. 1972. Culture of the virus of Gumboro on chicken embryo fibroblast. *Nuavo Veterinaria*, **48**: 195-201.

Tan, D.Y., Hair-Bejo, M., Omar, A.R. & Aini, I. 2004. Pathogenicity and molecular analysis of an infectious bursal disease virus isolated from Malaysian village chickens. *Avian Disease,* **48**(2): 410-416.

van den Berg, T.P. 2000. Acute infectious bursal disease in poultry: a review. *Avian Patholology*, **29**: 175-194.

Yamaguchi, T., Ogawa, M., Inoshima, Y., Miyoshi, M., Fukushi, H. & Hirai, K. 1996. Identification of sequence changes responsible for the attenuation of highly virulent infectious bursal disease virus. *Virology,* **223**: 219-23.