Replication of Very Virulent Infectious Bursal Disease Virus in the Chicken Mesenchymal Stem Cells

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ABSTRACT
The susceptibility of the chicken mesenchymal stem cells to very virulent infectious bursal disease (vvIBDV) was characterized after five consecutive passages in chicken mesenchymal stem cells. Virus replication was monitored by cytopathic effect observation, indirect immunoperoxidase, and reverse transcription polymerase chain reaction (RT-PCR). At 48 h post-infection (p.i.) in first passage, the cytopathic effect was characterized by rounding up of cells and monolayer detachment, intracytoplasmic brownish colouration was readily observed by from 24 h p.i onwards, viral RNA from vvIBDV-infected monolayers was demonstrated by RT-PCR. Tissue culture effective dose50 (EID50) was used to measure virus titration performed on chicken mesenchymal stem cells, and the titres in second passage was 10^6.6 EID50/ml. The results obtained in this study suggested that the chicken mesenchymal stem cells can be used for vvIBDV propagation, and this may trigger new aspects for tissue culture adaptation of such fastidious virus in the future.

Keywords: Poultry, very virulent infectious bursal disease virus, VP2, chicken mesenchymal stem cell

1. Introduction

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 immunosuppression (Van den Berg, 2000). The IBD virus (IBDV) can infect and grow on various primary cell culture of avian origin and certain cell line of mammalian origin. Commonly tissue culture used to replicate IBDV are; chicken embryo fibroblast (Lukert & Davis, 1974), chicken embryo kidney, baby hamster kidney (El-Febrary et al., 1997), ovine kidney (Kibenge & Mukenna, 1992), normal chicken lymphocytes, B-cell lymphoblastoid and rabbit kidney (Rinaldi et al., 1972), baby grivet monkey kidney and M4-104 cells (Jackwood et al., 1987), Vero cells are fibroblast like cells, its source is the kidney of a normal adult African green monkey (Peiñín et al., 1997). Isolation and propagation of 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 be due to
the strain differences in field viruses (Mannan et al., 2009). Recently, a long-term cell culture chicken mesenchymal stem cells have been established in our laboratory. These cells derived from bone marrow-mesenchymal stem cells and examined for its ability to support replication of the vvIBDV.

2. Methodology

2.1 Virus

The virus isolate (UPM0081), with an accession number of AY520910 was isolated during IBD outbreak in 2000 in Kelantan Malaysia (Tan et al., 2004). The isolate was passaged in specific-pathogen-free embryonated chick-eggs via chorioallantoic membrane (CAM) for 3 times, prior to adaptation in the chicken mesenchymal stem cells.

2.2 Cells and media

Bone marrow cells were harvested from the femur bones of 2-week-old chickens as described (Khatri and Sharma 2009). Briefly chicken mesenchymal stem cells were collected, washed with PBS and digested with trypsin/EDTA. The reaction was stopped by adding DMEM complete growth medium (GIBCO Laboratories, USA) supplement with; 2.0 g NaHCo3, 10% fetal calf serum (FCS) and 1% antibiotic of penicillin-streptomycin. After centrifugation at 1000g for 10 mins, the chicken mesenchymal stem cells were resuspended in the same medium and filtered through sterile gauze. The chicken mesenchymal stem cells in the filtrate were distributed on plastic tissue culture flasks and incubated at 37°C with 5% CO2. mesenchymal stem cells showed a fibroblast-like morphology and were subcultured prior to confluency. In this study, mesenchymal stem cells were used at passages 3–5

2.3 Extraction of viral RNA

The viral infected cell culture filtrate was used for viral RNA extraction and purification using Trizol reagent (Gibco BRL,Life Technologies) following the method recommended by the manufacturer.

2.4 Reverse transcriptase and polymerase chain reaction (RT-PCR)

In the RT step, 4µl of viral RNA was mixed with 1µl of DMSO, 100pg of each primer and 2 µl of distilled water, incubated for 5 mins at 95°C and chilled on ice. Then 0.5µl of AMV reaction buffer, 4µl of 10 mmol/l dNTPs, 4µl of 25 mmol/l MgCl2, 20 U of RNase, 0.5µl of AMV reverse transcriptase (promega) and dH2O were added to a final volume of 20µl. The reaction ran at 42°C for 1 hr and at 99°C for 1 min. The PCR step the primers p1 (5´-TCA CCG TCC TCA GCT TAC-3´, nt 587-604) and 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. 5µl of cDNA was mixed with 100pg of each primer, 6µl of Taq amplification buffer, 1µl of 10mmol/l dNTPs, 4µl of 25 mmol/l MgCl2, 2.5 U of taq DNA polymerase (promega) and distilled water to a final volume of 50µl. After initial denaturation at 95°C for 1 min, amplification proceeded in a DNA thermal cycler in 35 cycles
of denaturation (94°C/1min), primer annealing (48°C/1min) and primer extension (72°C/2min), with a final extension at 72°C for 10 mins.

2.5 Indirect Immunoperoxidase Staining Test

The indirect immunoperoxidase test (IIP) was done according to the method of Guvenc et al., 2004. The infected chicken mesenchymal stem cells were fixed with cold methanol: acetone (50:50 v/v) for 5 mins. The glass slides were then immersed in 1% H₂O₂ in absolute methanol for 30 mins. The PBS was then added to the glass slide for 15 minutes. The glass slides were then air dried. The hyper immune serum was diluted 1:1000 with PBS and added to the glass slide incubated for 1 hour in room temperature. The glass slides were then washed 3 times with PBS for 5 mins each. The rabbit anti-chicken IgG-HRP conjugated secondary antibody (Bio-Red, 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 minutes in a dark room. The slides were mounted with buffer glycerol and examined under light microscope.

2.6 Tissue culture infective dose 50 (TCID50)

The infectivity of replicate vvIBDV to chicken mesenchymal stem cells were determined by calculating 50% end point, as described by 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 microtiteration plate (Titertek, UK) was used to prepare chicken mesenchymal stem 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 37°C for 1 hour to allow adsorption. Then 100 μl of prewarmed maintenance medium was added in each well and again incubated at 37°C in 5% CO₂. The plate was observed twice daily for CPEs. The CPEs were stained with 1% crystal violet solution. The highest dilution of virus showing 50% CPEs was considered as end point to calculate TCID50

3. Results & discussion

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 chicken mesenchymal stem cells were formed following 24 hours of growth in DMEM growth medium. The morphology of normal chicken mesenchymal stem cells was fibroblast like (Fig 1). The vvIBDV strain (UPM0081) has been successfully, adapted in chicken mesenchymal stem cells with the formation of CPEs. The CPEs were observed in first passage after two days post inoculation (2dpi) while in passage 2 and 3 complete CPEs were recorded at 1 dpi (Fig1). The CPE in chicken mesenchymal stem 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 classical method of detecting the replication of virus in the cell culture using the cytopathic effect was augmented in this study with indirect immunoperoxidase and the viral antigen was observed as brownish intracytoplasmic granules in chicken mesenchymal stem cells (Fig 2). This finding is similar to the report of Guvenc et al., (2004).

The total infectious titer on chicken mesenchymal stem cells adapted vvIBDV (2nd passage) was found 106.6 TCID50/ml after 72 hours of infection. Kibenge et al. (1988) also reported similar findings, when they observed growth pattern of five strains of serotype 1 and 2 and variant strains of IBDV in Vero cells. They found titers ranged from 6.85 to 8.35 log10 TCID50/ml in Vero cell after 48 hours of infection, while from 5.35 to 6.10 log10 TCID50/ml in chicken embryo fibroblast (CEF) at 72 hours post-infection.

RT-PCR has been used widely as a rapid, sensitive, specific, and high throughput methodology for fast detection of genetic materials. This method was used successfully for detection of vvIBDV. In this study RT-PCR performed for viral RNA extracted from infected mesenchymal stem cells using P1 and P2 pair of primers specific to hypervariable region of VP2 gene resulted in generation of a targeted amplification of 643 bp. The use of this technique for virus detection has been reported previously (Tan et al., 2004).

Finally, the development of safe and reliable laboratory techniques that could isolate and propagate vvIBDV field strain in chicken mesenchymal stem cells may open a new opportunity to use this cell culture as a tool for routine diagnosis in the future.

Fig. 1. (A) Uninfected control chicken mesenchymal stem cells monolayer. (B) Cytopathic effect of UPM0081 isolate of the 1st passage at day 2 pi. The arrow shows cell rounding and clumping.
Fig. 2. Identification of IBD antigens in chicken mesenchymal stem cells culture using infected cell cultures stained with HRP-conjugated antibody. (A) Uninfected control chicken mesenchymal stem cells. (B) chicken mesenchyme stem cells infected with UPM0081 at 1st passage at day 1 pi. The arrow shows the presence of specific intracytoplasmic brownish colouration.

Fig. 3. Hypervariable region (643pb) of IBDV VP2 genes. Lane 1- positive 5 days post inoculation passage 1; Lane 2 positive 3 days post inoculation passage 2; Lane 3 Negative control; M- 100 bp DNA marker (Promega, USA).
4. Conclusion

The present study indicates that mesenchymal stem cell was a good alternative host system for isolation as well as propagation of vvIBDV from field bursa samples, indirect immunoperoxidase and RT-PCR could be successfully used to confirm the growth of vvIBDV in mesenchymal stem cells

References


