Original Article

Replication of Infectious Bronchitis Virus in the Chicken Mesenchymal Stem Cells

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ABSTRACT

The susceptibility of the chicken mesenchymal stem cells to infectious bronchitis virus was characterized after twenty consecutive passages in chicken mesenchymal stemm cells. Virus replication was monitored by cytopathic observation, indirect immunoperoxidase, and reverse transcription polymerase chain reaction. At 72 h post-infection (p.i.) in third passage, the cytopathic effect was characterized by rounding up of cell, monolayer detachment, intracytoplasmic brownish colouration was readily observed by from 24h p.i in third passage, and at all times the extracted viral RNA from IBV-infected monolayers was demonstrated by reverse transcription polymerase chain reaction. Tissue culture effective dose$_{50}$ was used to measure virus titration performed on chicken mesenchymal stem cells and the titres in twenty passages was 108.6 TID$_{50}$/ml. The results obtained in this study suggested that the chicken mesenhymal stem cells can be used for adaptation IBV and may be considered a step forward for the use of these cells in the future for IBV vaccine production.

Keywords: Infectious bronchitis disease virus, S1 gene, chicken mesenchymal stem cell

INTRODUCTION

Infectious bronchitis virus (IBV) infects the respiratory tract, kidneys and oviduct of chickens of all ages, causing retarded growth, mortality, reduced egg production and inferior egg shell quality and in many countries the disease remains one of the main problems affecting existing or developing poultry industries. Until now, there is no cure for the disease (OIE, 1996). Prevention is to import birds from disease-free flocks only or through vaccination, broilers are normally vaccinated at 1 day of age with live attenuated vaccines (Cavanagh & Naqi, 1997). In addition, breeders and egg layers are also vaccinated at approximately 8 week intervals with live attenuated vaccines, and with inactivated vaccines after they start laying eggs (Cook et al., 1999). Acute IBV are usually detected by the indirect immunoperoxidase test, enzyme-linked immunosorbent assay, virus isolation or serological approaches (de Wit et al, 1997; de Wit, 2000).

However IBV infections can also be diagnosed by detection of viral RNA, which make the diagnostic rapid and also dependable (Capua et al., 1999; Cavanagh, 2001). Usually, IB vaccines have been produced by growing vaccine virus strains in embryonated chicken eggs. IBV is harvested from the allantoic fluid and used to create a vaccine, nevertheless this method has the disadvantages of being labour-dense, takes long time and requires large area for the incubation of eggs. Cell cultures on the other hand considered are more suitable and less expensive than eggs and also convenient to inspect microscopically for indication of viral proliferation (Dhinakar & Jones, 1997; de Wit et al., 1997). There is therefore an urgent need to improve on the current IBV vaccines production technologies based on chicken-mesenchymal stem (CMS) cells. The development of cell-culture platforms as an alternative to the eggs for the manufacture of IBV vaccines is likely the most rapid and promising solution to overcome current vaccines production. In this study, firstly we investigated here for the first time the ability of IBV to grow in CMS cells and second to detect titres of IBV in CMS cells plus the possibility to use these cells for vaccine in the future

MATERIALS AND METHODS

Virus inoculation

The stock of IBV was originally obtained from the Faculty of Veterinary Medicine / University Putra Malaysia This virus was isolated from the allantoic fluid of embryonated chicken eggs. Initially, 0.3 ml of the virus stock was diluted in 30 ml of 10x PBS giving a dilution of 1:100. This diluted virus was used to inoculate CMS cells. Fully confluent 25 cm^2 flasks of

cells were used for virus passage in an attempt to adapt the virus to replicate in these cells.

Cells and media

Bone marrow cells were collected from the femur bones of 2-week-old chickens as described (Khatri & Sharma 2009). Shortly CMS cells were harvested, 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 CMS cells were resuspended in the same medium and filtered through sterile gauze. The CMS cells in the filtrate were distributed on plastic tissue culture flasks and incubated at 37°C with 5% CO_2. MCS cells showed a fibroblast-like morphology and were subcultured prior to confluency.

Tissue culture infective dose 50 ($TCID_{50}$)

The infectivity of replicate IBV to MCS cells were determined by calculating 50% end point, as described by Reed & Muench (1938). Ten-fold serial dilution of NDV was prepared in PBS from 10^{-1} to 10^{-10}. A 96 well tissue culture microtiteration plate (Titertek, UK) was used to prepare CMS 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% CO_2. 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 $TCID_{50}$

Indirect Immunoperoxidase Staining Test

The indirect immunoperoxidase test (IIP) was done according to the method of de Wit et al. (2000). The infected CMS cells were fixed with cold methanol: acetone (50:50 v/v) for 5 mins. The glass slides were then immersed in 1% H_2O_2 in absolute methanol for 30 mins. The PBS was then added to the glass slide for 15 mins. 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-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 minutes in a dark room. The slides were mounted with buffer glycerol and examined under light microscope.

RNA isolation

RNA was isolated from infected cells using commercial RNeasy Mini Kit (Qiagen, USA) as recommended by the supplier.

Reverse transcription polymerase chain reaction (RT-PCR)

For RT-PCR the infected monolayers were submitted to detect IBV replication, the partial spike (S1) gene (1025 nucleotides) of IBV was amplified RT-PCR after different passages according to the manufacturer's recommendation (Takara). The specific primers for IBV S1 gene were designed according to IBV H52 sequence (accession number AF3523151) as follows: forward, 5-CTATGTAGTG CTGTTTTG-3 (nucleotides 42 to 59); reverse, 5-CCTTGAAGAGG ACCGTAA-3 (nucleotides 1049 to 1066), and the RT-PCR was run 30 min at 50^0C and 2 min at 94^0C for one cycle, then 30 cycles of 30 sec at 94^0C, 30 sec at 55^0C, 1.5 min at 72^0C, followed by 15 min at 72^0C (PTC-100TM Programmable Thermal Controller; MJ Research, Inc.).

Detection of PCR products

PCR products were separated in 1.5% agarose gel in 1 x TAE buffer stained with ethidium bromide, compared with molecular mass marker and visualized by ultraviolet (UV) transillumination.

RESULTS

Cytopathic effect (CPE)

Infected cell monolayer steadily became broken as the virus proliferates to occupy extra cells in culture. In the first and second passage, the infectivity was sluggish and not very clear as the virus was just begin to adapt on cells. Through the third passage, we were able to visualize CPE but it was not whole the cells. During the fourth and fifth passage, CPE was rapid and showed in two days. It was characterised by rounding of cells, failure of adhesion, vacuolization in cells, clustering of infected cells (Figure 1).

TCID50

The infectivity titre of the virus was found to increase gradually from the 5th to the 20th passage. The $TCID_{50}$ titre was 8.6 after 20 passages (Table I)

Table1. Virus titer determined by Tissue Culture Infective Dose$_{50}$ ($TCID_{50}$)

Virus category and passage level	Virus titre $TCID_{50}$/ml
Virulent-unadapted	–
5	6.5
10	7.4
15	8
20	8.6

Indirect Immunoperoxidase Staining Test (IIPS)

In IIPS, brown complexes were seen as brownish intracytoplasmic granules around the nucleus (Figure 2) in infected chicken mesenchymal stem cells cultures treated with IBV antiserum, but such effects was not seen in control cell cultures

RT-PCR

RT-PCR of different passages at passages 2, 5, 8, 10, 13, 15 and 20 were performed. The expected 1025 bp was obtained for each passage examined (Figure 2).

DISCUSSION

Infectious bronchitis (IB) virus, early described in 1930 (Schalk & Hawn, 1931), continues to be a main cause of disease in chickens of all ages and types in all parts of the world (Anon, 1991). The disease is occurring in all countries with a concentrated poultry industry, with the occurrence of infection close to 100% in most locations (Ignjatovic & Sapats, 2000). Most of the countries rearing poultry commercially rely on vaccination to control IB, but the vaccine is still a great challenge for these countries because of the wide variety serotypes for this virus. A large majority of the vaccines available are chick embryo–adapted vaccines. Cell cultures provide a useful alternative system for the virus preparation and adaptation, and find a suitable cell culture for IBV (Myint, 1994).

In the present study, attempts were made to adaptation IBV isolate by blind passage first in SPF embryos (Gelb & Jackwood, 1998) and then adapted to the CMS cells until 20th passages. The cytopathic effects (CPEs) appeared after 72 hours of infection in third passage and this observation was also noticed by (Hopkins, 1974), but slightly varied from findings of Mahgoub et al. (2010) where the IBV was adapted to Vero cell line after the third passage. The difference might be due to the cell culture passage level of the virus strains used or variation in sensitivity of cell culture to different strains.
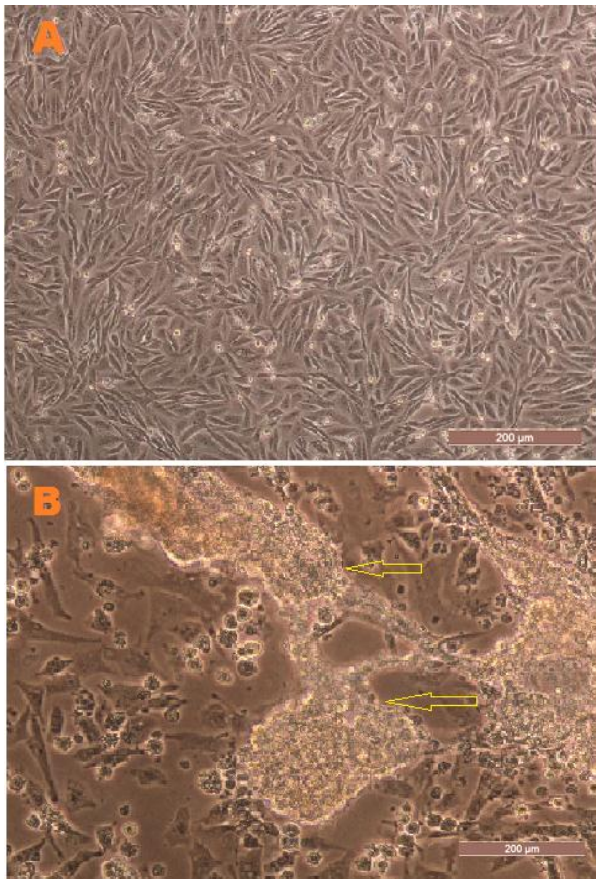
The total infectious titer passage 20 was found to be $10^{8.6} TCID_{50}/mL$. These finding supports the previous report by (Otsuki et al., 1979) they found titer $10^9 TCID_{50}/mL$ in passage 25 in Vero cells.

The classical method of detecting the adaptation and replication of virus in the cell culture using the cytopathic effect was augmented in this study with indirect immunoperoxidase is a relatively inexpensive and rapid (Arshad & Salihi 2002). Monoclonal antibody (Mab) reacting only with one or small number of epitope (s) of the IBV antigen, results in viral antigen was observed as brownish intracytoplasmic granules in mesynchymal stem cells (Arshad & Salihi 2002).

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 IBV. In this study RT-PCR performed for viral RNA extracted from infected CMS cells to amplify of 1025 bp. The use of this technique for virus detection has been reported previously (de Wit et al., 2000).

We can conclude from this study that CMS cells have used for the first time to isolate and adapt IBV successfully which could open new horizons to use these cells for the vaccine production in the future.
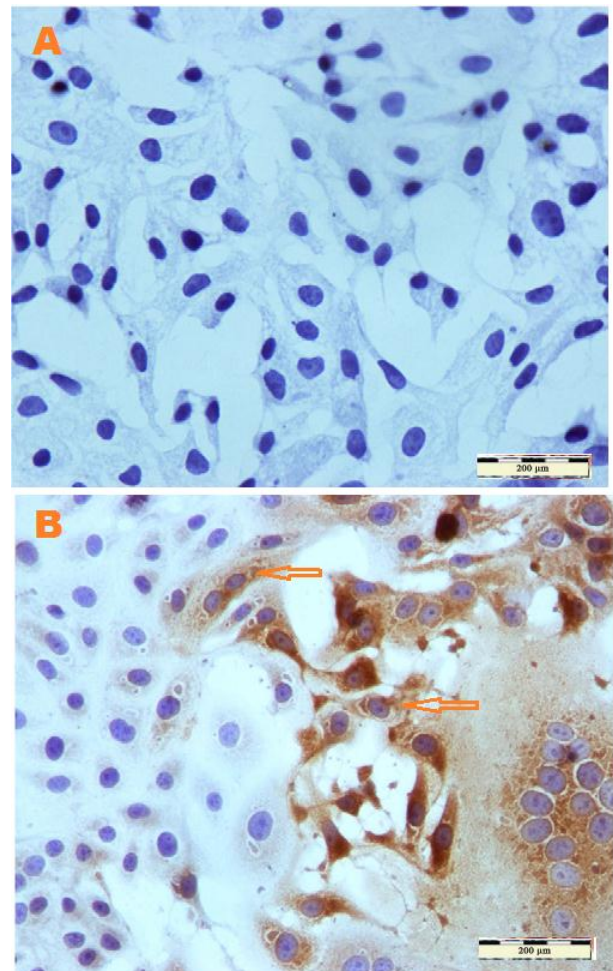

Figure 1. (A) Uninfected control CMS cells monolayer. (B) Cytopathic effect of IBV isolate of the 3rd passage at day 5 pi.
The arrows show detachment of cells from the substrate with the eventual destruction of the entire monolayer. 10 x. Bar = 200 μm

Figure 2. Identification of IBV in CMS cells culture using infected cell cultures stained with HRP-conjugated antibody. (A) Uninfected control CMS cells. (B) CMS cells infected with IBV at 3rd passage at day 5 pi. The arrow shows the presence of specific intracytoplasmic brownish coloration. 10x. Bar = 200μm
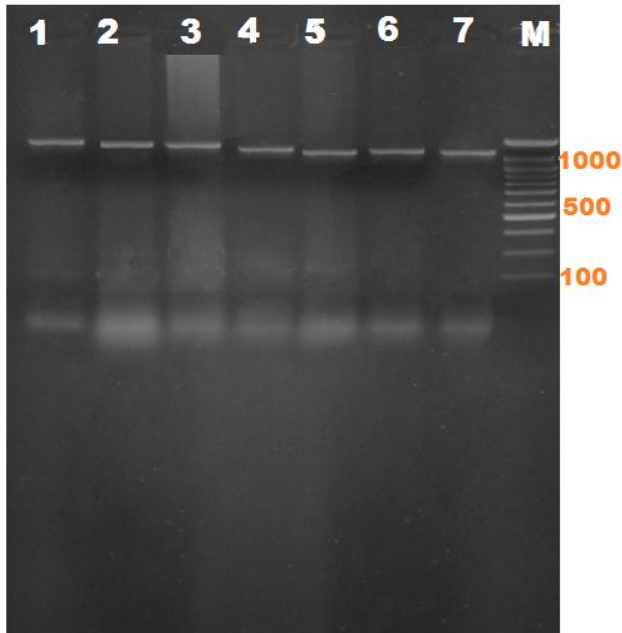
Figure 3. S1 gene (1025 pb) of IBV. Lane 1 positive passage 2; Lane 2 positive passage 5; Lane 3 positive passage 8: Lane 4 passage 10; Lane 5 positive passage 13; Lane 6 positive passage 15 and Lane 7 positive passage 20; M- 100 bp DNA marker (Promega, USA).

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