ADAPTATION OF AN INDIGENOUS VERY VIRULENT INFECTIOUS BURSAL DISEASE VIRUS ON VERO CELL LINE

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ABSTRACT

In the present study, Vero cell line was tested for its ability to support the replication of indigenous very virulent infectious bursal disease virus (vvIBDV). The frozen cells were resuscitated to prepare monolayer, which was further sub-cultured to prepare semi-confluent monolayers using M199 growth medium supplemented with 5% foetal calf serum. The semi confluent monolayers were then infected with 0.25 ml of indigenous vvIBDV. The passage 1 virus was harvested and used for the next passage. In this way virus was given three serial passages on Vero cell line, where characteristic cytopathic effects (CPEs) were observed. During the first passage, no CPEs were found. The Vero cell monolayers remained normal in first passage upto 144 hours post-infection. During second passage, rounding of cells was observed after 72 hours of infection. However, clear and consistent CPEs were not observed in 2nd passage. Typical aggregation, rounding and granulation of Vero cells was noticed in passage 3 (P3) from 72 hours upto 144 hours post-infection. The positive results of agar gel precipitation test (AGPT) confirmed that the adapted (P3) virus was IBDV. The infectivity titer of adapted vvIBDV was found to be $\log_{10}$ 7.60 $TCID_{50}$/ ml at 72 hours post-infection. The indigenous vvIBDV was well adapted to Vero cell line after three successive passages.

Key words: Very virulent, Infectious bursal disease virus, Vero cell line; cytopathic effects.

INTRODUCTION

The traditional isolation method for infectious bursal disease virus (IBDV) using the chorio-allantoic membrane of 9 to 11 day old chicken embryos (Hitchner, 1970) is no longer reliable, as some variant strains of the virus cause no embryo mortality (Rosenberger and Cloud, 1986). Many IBDV isolates have been adapted to primary cell cultures of chicken embryo origin, including chicken embryo kidney, chicken embryo bursa and chicken embryo fibroblast (CEF) cells (Raymond and Hill, 1979; Yamaguchi *et al.*, 1996). These cells produce low yield of virus and have limited growth properties (Lukert *et al.*, 1975). So there is a need for cell cultures that can produce higher yields of infectious virus required for experimental purposes.

Mammalian continuous cell lines reported to be susceptible to IBDV include RK-13 derived from rabbit kidney (Rinaldi *et al.*, 1972), Vero cells derived from adult African green monkey kidney (Jackwood *et al.*, 1987; Kibenge *et al.*, 1988; Peilin *et al.*, 1997; Ahasan *et al.*, 2002), BGM-70 derived from baby grivett monkey kidney (Jackwood *et al.*, 1987), MA-104 derived from foetal rhesus monkey (Jackwood *et al.*, 1987) and OK derived from ovine kidney (Frederick *et al.*, 1992). The use of these cell lines has several advantages over the primary cell culture of avian origin. These are easy to handle and maintain and free from vertically transmitted extraneous viruses of avian origin (Hassan *et al.*, 1996).

If higher viral titers could be obtained, continuous cell line would be valuable and economical method of growing virus. It would be useful for laboratories that have limited access to specific-pathogen-free (SPF) embryos for CEFs or for SPF chicks as we have in Pakistan. The present study was designed to investigate the usefulness of the continuous Vero cell line for the adaptation of indigenous very virulent IBDV (vvIBDV).

MATERIALS AND METHODS

Source of virus and Vero cell line

The locally isolated and characterized vvIBDV was obtained from the Department of Veterinary Microbiology, University of Agriculture, Faisalabad. Adult African green monkey kidney cells (Vero cell line) were imported from Centre for Applied Microbiology and Research in the form of frozen ampoule (ECACC No. 84113001, ECACC; Salisbury, Wiltshire, UK). The ampoule was placed immediately in gaseous phase of liquid nitrogen cylinder at -196°C until used.

Preparation of Vero cell monolayer

Frozen ampoule was taken out of liquid nitrogen cylinder and cells were resuscitated in water bath at 37°C for 2 minutes. The ampoule was transferred to class-II safety cabinet (Telstar, Spain) and cells were drop wise pipetted into a 25 cm^2 tissue culture flask (Nunc Easyflasks, Sigma) having 5 ml prewarmed M199 growth medium with 5% fetal calf serum (FCS). The flask was incubated at 37°C in the presence of 5% of CO_2 in CO_2 incubator (Sanyo, Japan). The cells were examined twice daily under inverted microscope (Olympus CK40, Japan) for the formation of complete monolayer.

Sub-culturing of adherent monolayer

Confluent monolayer was brought into suspension using prewarmed 0.25% trypsin/EDTA solution. The viability of cells was checked with trypan blue stain. The viable cells were counted and reseeded into new flasks containing prewarmed M199 growth medium @ $3x10^4$ cells/cm^2. In the same way, sub-culturing was performed when needed.

Infection of Vero cell monolayers

Normal and semi-confluent monolayers of Vero cells at 24 hours after sub-culturing were used for infection with vvIBDV, as described by Ahasan *et al.* (2002). The growth medium of each flask was removed and cell monolayer was washed twice with prewarmed phosphate buffered saline (PBS). Then the Vero cells were infected by 0.25 ml of IBDV using 0.2 µm pore size filter (Whatman International Ltd., England). The inoculum was spread uniformly over the monolayers and incubated at 37°C for 1 hour with intermittent rotation to allow adsorption. One flask was kept as un-inoculated control. Five ml of maintenance medium (M199 medium with 2% FCS) was added to each flask. The flasks were incubated at 37°C in 5% CO_2 and monolayers were examined twice daily under inverted microscope for cytopathic effects (CPEs).

Harvesting of virus

The culture supernatant was harvested by three freeze-thaw cycles, as described by Peilin *et al.* (1997). The infected flasks were transferred to -20°C for over night at 144 hours post inoculation and then thawed at room temperature. This process was repeated three times. The virus suspensions were poured into appendorf tubes and centrifuged at 5000 rpm for 5 minutes at 4°C to pellet the cell debris. The clear supernatant fluid was collected carefully, divided into two aliquots, labeled as passage 1 (P1) and stored at -70 °C till further use.

Adaptation of Virus

The passaged (P1) virus was inoculated again to fresh monolayers of Vero cells using the same technique and observed for CPEs. The virus was harvested at 144 hours post-inoculation by three freeze-thaw cycles, clarified by centrifugation and labeled as passage 2 (P2). Similarly passage 3 (P3) virus was obtained through 3rd infection and CPEs were observed twice daily upto 144 hours post-inoculation.

Identification and confirmation of virus

The IBDV was identified on Vero cells through its CPEs. The characteristic changes in infected monolayers were carefully examined in each passage. The time for the appearance and intensity of CPEs were also recorded in each passage. To confirm that the adapted virus on Vero cells was IBDV, the supernatant stored from each passage was subjected to agar gel precipitation test (AGPT) (Cullen and Wyeth, 1975), using known hyperimmune serum (Hussain *et al.*, 2004).

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

The infectivity of adapted IBDV to Vero cells was determined by calculating 50% end point, as described by Reed and Muench (1938). Ten-fold serial dilution of IBDV was prepared in PBS from 10^{-1} to 10^{-10}. A 96 well tissue culture microtiteration plate (Titertek, UK) was used to prepare Vero cell 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 $TCID_{50}$.

RESULTS AND DISCUSSION

Vero cells are derived from normal adult African green monkey kidney. This cell line is being used world wide to grow and propagate a number of viruses of avian and non-avian origin. In Pakistan, there are very virulent strains of infectious bursal disease virus (vvIBDV) as characterized through reverse transcriptase - polymerase chain reaction (RT-PCR) and restriction fragment length polymorphism (RFLP) techniques (Zahoor *et al.*, 2005).

Normal and confluent monolayer of Vero cells was formed following 48 hours of growth in M199 growth medium. The morphology of normal Vero cells was fibroblast like (Figure 1). Kibenge *et al.* (1988) also used M199 growth medium for the growth of serotype 1 and 2 and variant strains of IBDV in Vero cells. They also reported that complete monolayer of Vero cells was formed within 24 to 48 hours after culturing.

During the first passage of IBDV on Vero cell line, the indigenous virus did not produce any evidence of cytopathic effects (CPEs). The monolayer remained 100% intact in passage 1 (P1) upto 6 days (144 hours) post-infection. The virus just started to adapt on Vero cell line and of virus infectivity to Vero cells was low. The P1virus was more virulent to Vero cells.

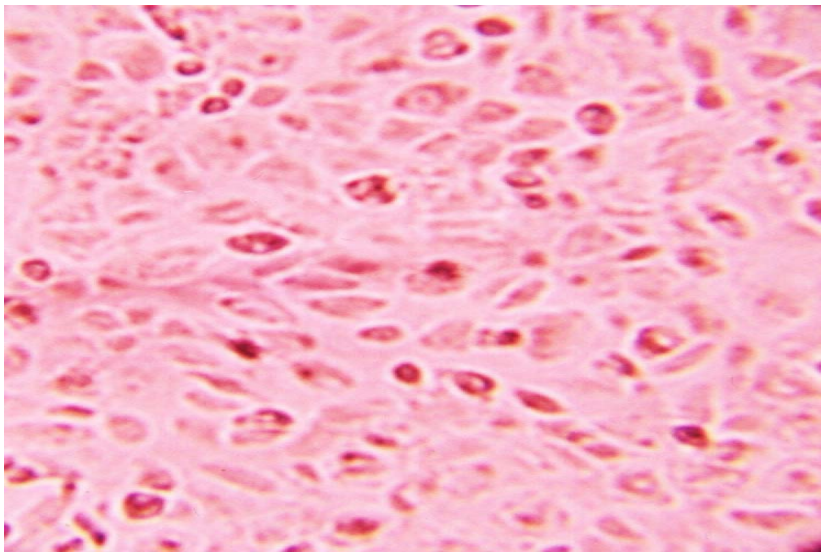

Fig. 1: Confluent monolayer of Vero cells grown in M199 growth medium (100x)

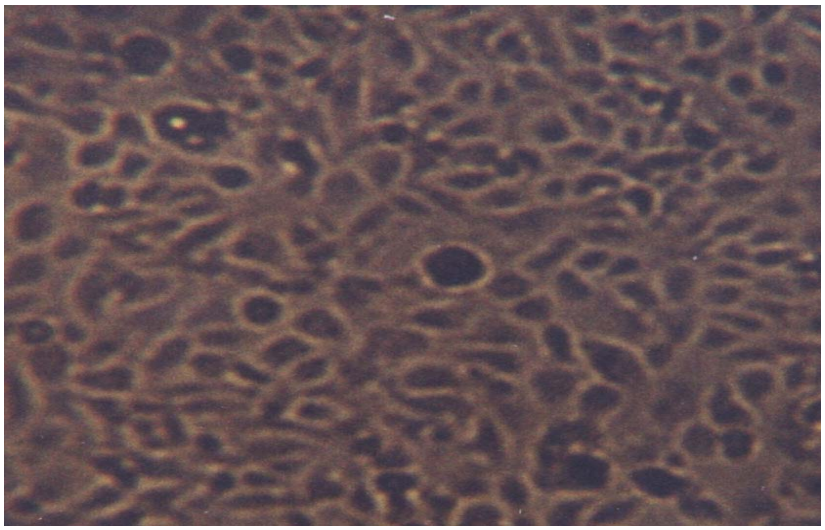

Fig. 2: CPEs of IBDV on Vero cell monolayer (Passage 2) 120 hours post-infection (100x)

During second passage, some changes in Vero cell monolayer began to develop after 72 hours of incubation following infection. Monolayer showed rounding of infected cells. Nevertheless, complete CPEs of IBDV on Vero cells were not found in this passage too. Clear and consistent CPEs of vvIBDV were found during third passage. The Vero cells were almost normal at 24 hours post-infection. The cells started to aggregate at 48 hours and rounding of cells started at 72 hours post-infection. The complete rounding of infected cells was observed at 120 hours post-infection. The granulation of cells was seen at 144 hours post-infection. The aggregation of cells, formation of round refractile cells and finally the granulation was the sequence of changes in third passage following infection with vvIBDV (Fig. 2).

These findings are in agreement with those of Peilin *et al.* (1997). They observed complete CPEs of IBDV on Vero cell line at 65 to 72 hours of infection during 4th passage. Ahamed *et al.* (2004) observed CPEs of Newcastle disease virus (NDV) on Vero cell line following 36 to 40 hours of infection during 3rd

passage. The CPEs of reovirus was also observed by Islam (1999) following 36 hours of infection in 3rd passage. Ahasan *et al.* (2002) observed the clear and consistent CPEs of IBDV on Vero cells at 144 hours post-infection during 3rd passage.

After the identification of adapted virus through characteristic CPEs, the virus from each passage was subjected to agar gel precipitation test (AGPT). Clear precipitation lines were obtained between known hyperimmune serum and virus from each passage. Ahasan *et al.* (2002) also used AGPT for confirmation of IBDV adapted on Vero cell line. The total infectious titer of Vero cell adapted vvIBDV (3rd passage) was found to be $\log_{10}$ 7.60 $TCID_{50}$/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 $\log_{10}$ $TCID_{50}$/ml in Vero cell after 48 hours of infection, while from 5.35 to 6.10 $\log_{10}$ $TCID_{50}$/ml in chicken embryo fibroblast (CEF) at 72 hours post-infection.

The present study reports the adaptation of Pakistani vvIBDV to Vero cells after three successive passages. Further study to determine the attenuation of vvIBDV for the vaccine production and mass production of test antigen for use in diagnostic tests is underway.

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