ISOLATION OF FOWL ADENOVIRUS IN CHICKEN EMBRYO LIVER CELL CULTURE AND ITS DETECTION BY HEXON GENE BASED PCR

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ABSTRACT
Inclusion body hepatitis is a sporadic disease condition caused by several serotypes of fowl adenoviruses. In the present study, Indian isolates of fowl adenovirus were propagated in primary chick embryo liver cell culture and the virus was detected by hexon gene based polymerase chain reaction. 10-13 day old chicken embryos were dissected to remove the liver and the liver tissue was subsequently trypsinised and processed for the preparation of chicken embryo liver cell culture. Filtered liver homogenates from dead chickens suspected for avian adenovirus infection was used as the virus inoculum for infecting the chicken embryo liver cell monolayers. The virus was subsequently passaged and the cytopathic effects were detected from the 2nd passage onwards. The cytopathic effects observed were in the form of rounding, clumping and detachment of cells subsequently leading to detachment of the entire monolayer at 96 hrs of infection. The growth of the virus was detected by polymerase chain reaction based on hexon gene and the cell culture supernatant from seventh passage of each isolate gave the specific PCR product proving the ability of chicken embryo liver cell culture to be used as a suitable medium for the isolation and propagation of fowl adenoviruses.

Key Words: Inclusion Body Hepatitis, Fowl Adenovirus, Primary Chicken Embryo Liver Cell Culture, Hexon Gene and Polymerase Chain Reaction

INTRODUCTION
Fowl adenovirus (FAV) is a member of the family Adenoviridae, infecting chickens and responsible for a sporadic disease condition called Inclusion Body Hepatitis (IBH). In India, IBH was first reported in 3-weeks old broiler chicks, characterized by enlarged, mottled and friable liver with intranuclear inclusion bodies in the hepatocytes, causing about 15% mortality (Grewal et al., 1981). IBH along with hydropericardium syndrome (IBH-HPS), popularly called ‘litchi heart’ disease, has been reported to be particularly important in some countries in Asia and America (Shane, 1996).

Isolation of the virus is an important and essential procedure for the subsequent identification and typing of the virus. The fowl adenovirus can be grown in primary cell cultures of chicken kidney (Khawaja et al., 1988) or chicken embryonic liver (CEL) cells (Naeem et al., 1995). CEL culture was used for the isolation of FAVs taken from liver tissues of birds suffering from IBH-HPS from different farms of India (Kataria et al., 1996). Embryonated eggs have also been reported as a sensitive medium for the isolation and propagation of FAVs, by inoculating liver homogenate of HPS affected birds in 10–12 day old specific pathogen free (SPF) chicken embryos through chorioallantoic sac route (Cowen and Braune, 1988; Abe et al., 1998) and 5-7 day old SPF chicken embryo via yolk sac route (Cowen and Braune, 1988; Mazaheri et al., 1998). In the present study, four Indian isolates of fowl adenovirus were propagated in primary chick embryo liver cell culture and the virus was detected by hexon gene based polymerase chain reaction.

MATERIALS AND METHOD
Samples for virus isolation
Liver samples were collected from dead chickens brought for post-mortem examination and suspected for avian adenovirus infection, in the Department of Veterinary Public Health and Epidemiology, Chaudhary Charan Singh Haryana Agricultural University, Hisar during the period 2009-2010. The tissues were collected aseptically in sterile vials containing 50% phosphate buffered glycerol and the vials were stored at -20 °C until further processing.

Detection of FAV hexon gene
For detection of FAV genes, 10% homogenates of the chicken liver tissues were prepared in lysis buffer. DNA was extracted from the homogenates using conventional phenol: chloroform: isoamyl alcohol (PCI) method as per the procedure recommended by Sambrook and Russel (Sambrook and Russell, 2001). For PCR H1/H2 primers published by Raue and Hess (Raue and Hess, 1998), was used which can detect DNA with 1,219 bases from whole
hexon gene of FAV. Nucleotide sequence of the forward primer (H1) was 5’TGGACATGGGGGCGACCTA3’ and that of the reverse primer (H2) was 5’AAGGGATTGACGTTGTCCA3’. PCR was done with Taq DNA polymerase (Fermentas) under conditions of initial denaturation at 94 ºC, 5 min followed by 35 cycles of 45s at 94 ºC, 1m at 60 ºC and 1min at 72 ºC, and final extension at 72 ºC, 10min. The PCR products were detected by gel electrophoresis with 1% Ultra Pure agarose (Invitrogen, USA) and 1 Kbp DNA ladder (Fermentas). Four samples which proved positive for avian adenovirus were used for the preparation of inoculum for propagation of virus in CEL culture.

**Preparation of chicken embryonic liver cell culture**

Primary chicken embryo liver (CEL) cell culture was prepared from 10-13 days old embryonated chicken eggs as per the method of Adair et al., (1979) with minor modifications. Liver from the embryos were removed aseptically and washed twice with Hanks Balanced Salt solution (HBSS) (pH 7.4). The liver tissue was minced and washed gently with HBSS and trypsinized gently with 0.25% trypsin-EDTA solution at 37 ºC for 30m. The trypsinised cells were decanted and filtered through sterile gauze tied to a sterile beaker. To stop the activity of residual trypsin, growth medium, DMEM (Sigma) containing 10 % foetal calf serum (FCS) (Sigma) was added to the filtrate at the rate of 10 ml/100 ml. The filtrate containing cells was centrifuged at 1500 rpm for 20 minutes at 4 ºC and the cell pellet obtained was washed twice in the growth medium. The live cell concentration was adjusted to 5 × 10^6 cells/ml of the medium and cell suspension in 10 ml and 5 ml volumes were seeded in 75 cm² and 25 cm² tissue culture flasks respectively and incubated at 37 ºC in BOD incubator until confluent monolayer formed.

**Isolation of the virus in CEL culture**

20% suspension (w/v), of liver homogenate in phosphate buffered saline (PBS), pH 7.4, containing antibiotics (penicillin 1000 IU/ml and streptomycin 1000 µg/ml) was prepared and subjected to 3 cycles of freezing and thawing and centrifuged at 1500 rpm for 20 minutes at 4 ºC. The supernatant obtained was filtered through 0.22µm membrane filter (Millipore, USA). The filtrate was stored in sterile vials at -20 ºC and used as inoculum for infection of the primary chicken embryo liver cell culture.

Flasks containing complete monolayers were washed twice with HBSS and 500µl and 1ml of the liver tissue homogenates were inoculated in 25 cm² and 75 cm² tissue culture flasks respectively. The virus inoculum was allowed to adsorb onto the cells at 37ºC for 60 min. Following adsorption, maintenance medium containing 2% FCS was added to the culture and the infected flasks were incubated at 37ºC and the monolayer was observed for cytopathic effect (CPE) daily for about three days. After CPEs developed prominently, the cells were frozen and thawed 3 times and viral fluid was harvested by centrifugation at 2,000 rpm, 10 min and stored at −20 ºC for further use and propagated FAVs was confirmed by PCR for hexon gene.

**Confirmation of virus propagation by hexon gene based PCR**

DNA was extracted from the cell culture supernatant using conventional phenol: chloroform: isoamyl alcohol (PCI) method as per the procedure recommended by Sambrook and Russel (2001) with minor modifications. To confirm the presence of virus in the cell culture supernatant, the extracted products were amplified by polymerase chain reaction using primers H1/H2. The PCR products were detected by gel electrophoresis with 1% Ultra Pure agarose (Invitrogen, USA) and 1 Kbp DNA ladder (Fermentas).

**RESULTS AND DISCUSSION**

The FAV produced CPE in chick embryo liver cell culture from the 2nd passage onwards. The cytopathic effects observed were in the form of rounding, clumping and detachment of cells, where as no such changes were observed in the uninfected CEL cell cultures. Swelling and rounding of the infected cells appeared within 48 hrs of infection and by 72 hrs post infection, the cells started detaching from the monolayer. Complete detachment of the monolayers observed at 96 hrs of infection. Subsequent passages gave CPE within 24 to 48 hrs and involved majority of the cells in monolayer. The microphotograph of multiplying liver cells and monolayer of CEL cell culture showing CPE are given in Fig. 1 and 2 respectively. Cell culture supernatant from seventh passage of each isolate was subjected to DNA extraction and PCR amplification using primer pair H1/H2 and a specific product of 1219 bp was detected by 1% agarose gel electrophoresis (Fig. 3). This confirmed the growth of the virus in the cell culture.

Many workers have attempted isolation of fowl adenovirus in CEL cell culture and succeeded in the adaptation of the virus (Oberoi et al., 1996; Kataria et al., 1997; Barua and Rai, 2003). In the present study, the FAV was propagated in primary chick embryo liver cell culture and the virus produced rounding, degeneration and detachment of the infected cells from the monolayer, similar to the observations of many earlier workers (Adair et al., 1979; Oberoi et al., 1996; Kataria et al., 1997; Balamurugan et al., 2002). The cell culture as template fluid was used to amplify 1219 bp FAV DNA product and this confirmed the virus in infected cell culture fluid.
CONCLUSION
The better growth of FAV in CEL cell culture may be attributed to the fact that the predilection site of FAVs is the nucleus of hepatocytes. The present study proves that the CEL cell culture is a very suitable medium for the isolation and propagation of FAVs.

REFERENCES


