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*Research Article*

# IDENTIFICATION OF SUITABLE CULTURE SYSTEM FOR FIELD FOWL ADENOVIRUS ISOLATION

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**ABSTRACT: The Present study was aimed to find out the suitable culture system for field fowl adenovirus isolation. Liver tissues were collected from fowl aviadenovirus suspected commercial broiler flocks (n=100 flocks) showing inclusion body hepatitis (IBH) lesions. For molecular characterization, polymerase chain reaction (PCR) was carried out for 897bp hexon gene of fowl adenovirus and amplified products were subjected for sequencing. All the PCR positive samples were screened for concurrent infection against marek's disease virus (MDV), chicken anaemia virus (CAV), reticular endothelial virus (REV) and avian leucosis virus (ALV). Field fowl adenovirus isolates free from concurrent infections (14 nos) were subjected for virus isolation using chorioallantoic route inoculation of embryonated SPF chicken eggs, *in vitro* infection of chicken embryo fibroblast (CEF) cells and chicken embryo liver cells (CELi). There is no embryo death and specific lesions on CAM were found in the inoculated embryonated egg up to five passages. Only nine out of fourteen PCR positive liver tissues produced cytopathic effects (CPE) in CEF culture at 72 h of 5$^{th}$ passage. All the fourteen isolates produced prominent CPE in CELi cells from 48 to 72 hrs of post infection at first passage level.**

**Keywords:** Fowl adenovirus isolation, Embryonated chicken eggs, Chicken embryo fibroblast culture, Chicken embryo liver cells.

## INTRODUCTION

Fowl adenoviruses (FAdVs), belonging to the genus *Aviadenovirus* and *Adenoviridae* family and have been grouped into five species based on their molecular structure and further divided into 12 serotypes and several strains in each serotype based on cross-neutralization assays [1, 7]. FAdV infection in chicken is endemic in India, and most notable disease associated with fowl adenovirus infection in chicken are inclusion body hepatitis (IBH), hepatitis-hydropericardium syndrome (HHS) and gizzard erosions (GE). Almost all and the most importantly serotype 11 of FAdV are involved in causing IBH in chicken in India. [2]. Voluminous evidence showed that, without influence of predisposing factors FAdV cause IBH in chickens [3, 4]. Diagnosis of FAdV infection can be carried out conventionally as well as molecular methods, but isolation of the virus is gold standard. For isolation of FAdVs from field outbreaks using SPF chicken eggs, chicken embryo fibroblast culture and chicken embryo liver / kidney cells are used but not all serotypes of FAdVs are multiplied and adopted in embryonated egg and cause distinguishable lesions [5]. Fowl adenovirus isolation can be made in various cell cultures, different routes of embryonated chicken egg inoculation and cell lines [6, 7]. Hence present study was aimed to find out suitable culture system for isolation of fowl adenovirus from field outbreaks.

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## MATERIALS AND METHODS

### Sample collection and processing

Sample such as liver tissues were collected from commercial broiler flocks (n=100) that showed reduced feed intake, dullness, increased mortality, depression, ruffled feathers, enlarged liver, and diarrhea. The samples were kept in sterile vials containing sterile phosphate buffered saline (PBS) pH 7.0 -7.4 with antibiotics (penicillin G - 100 units, streptomycin- 100 µg and 0.25 µg amphotericin B / ml) and stored at -800C up to processing. Liver samples were triturated, 20 % tissue suspension was centrifuged at 3000 rpm for 10 minutes and supernatant was used for virus isolation and also DNA extraction for PCR.

### Molecular screening by PCR

Liver tissues were subjected to DNA extraction using a commercial Qiagen DNA extraction kit. The DNA concentration and purity were assessed by NanodropTM. FAdV hexon gene specific forward primers F- 5' CAARTTCAGRCAGACGGT 3' and reverse primers R- 5'TAGTGATGMCGSGACATCT 3' was used for DNA amplification [8]. The PCR cycle condition used in this study was: Initial denaturation of 94°C for 10 min; Denaturation of 94°C for 5min; Annealing of 62°C for 30 sec and Extension of 72°C for 2 min; 35 cycles followed by final extension of 72°C, 10 min. The amplified products were subjected for agarose gel electrophoresis with 1.5% agar and, stained with ethidium bromide to identify amplicon size of 897 bp Gel documentation system (Bio Rad, USA). The same DNA was subjected to screen the co viral infections of FAdV *viz.*, Chicken anaemia virus (CAV), Reticular endothelial virus (REV), Marek's disease virus (MDV) and, Avian leucosis virus (ALV). The FAdV isolates which were free from above co viral infections were used for virus isolation.

### Isolation of FAdV using embryonated SPF chicken egg through chorioallantoic membrane

Polymerase chain reaction positive and antibiotic treated liver tissue supernatants were filtered through a 0.45 µm Millipore membrane filter and used for virus isolation. The 0.2 ml of clear liver supernatant was inoculated aseptically through the chorioallantoic membrane route (CAM) in 11 days old embryonated SPF chicken eggs and incubated at 37ºC for five days. After five days of post inoculation live embryos were chilled overnight at 4ºC, and chorioallantoic membrane (CAM) and liver tissues of the embryos were harvested aseptically, and up to five such passages were carried out. The embryos of each passage were examined for mortality, pale, friable, and enlarged liver and congestion and cloudiness of CAM. Liver tissues and CAM were collected from all five passages and confirmed by PCR before declaring it as negative for FAdV.

### Isolation of FAdV using primary chicken embryo fibroblast cell culture

Nine to eleven days old SPF chicken embryos were used for preparation of primary chicken embryo fibroblast cell culture [9]. Embryos were aseptically lifted from egg, and head, limbs and viscera of the embryos were removed with sterile forceps and discarded and washed thrice with Hank's basal salt solution (HBSS). Trypsinization was carried out with trypsin-EDTA solution (0.25%) at 37˚C for 20 min. Residual trypsin activity was stopped by addition of chicken embryo fibroblast medium (Hi-media) with 10 per cent foetal calf serum. To obtain confluent monolayer, chicken embryo fibroblast medium (Himedia) with 10 % foetal calf serum seeded with $10^6$ cells /ml and dispensed into tissue culture flasks and, incubated for 48 h at 37°C with 5% $CO_2$ and 75% humidity in incubator. The liver tissues positive for the hexon gene of FAdV by PCR were used for inoculation of CEF monolayer. The infected monolayers were observed every day for the development of CPE for seven days. Five passages were carried out and the harvest was further confirmed by FAdV hexon gene specific PCR, before declaring it as negative for FAdV isolation.

### Fowl adenovirus isolation of using chicken embryo liver cells

Thirteen to fifteen days old SPF chicken embryos were used for preparation of chicken embryo liver cell culture (CELi) with slight modifications in trypsinization time [10]. Embryos livers were removed aseptically and washed several times with HBSS to remove excess blood. Aseptically, liver tissues were minced and trypsinization was carried out by adding 10 ml of 0.25 per cent trypsin and stirring thoroughly at 37°C for 15 min. The whole suspension was transferred onto a sterile muslin cloth covered beaker. Residual trypsin activity was stopped by the addition of growth medium consisting of Dulbecco's Modified Eagle Medium with high glucose (Hi-media) and 10 % foetal calf serum. The concentration of the cells was adjusted to 3 x$10^6$/ ml of growth medium, dispensed into tissue culture flasks and incubated for 48 h at 37°C in an incubator with 5% $CO_2$ and 75 per cent humidity to obtain confluent monolayer. The confluent monolayer in

disposable tissue culture flasks was inoculated with 200 µl of sterile inoculum and incubated for 2 to 3 days for the development of intensive cytopathic effect (CPE). Up to five passages were carried out and the harvest was confirmed by hexon gene specific PCR, before declaring it as negative for FAdV isolation.

**Identification of fowl adenovirus isolates by hexon gene specific PCR**

The screening of field isolates from embryonated chicken eggs, CEF and CEli cell culture was performed with PCR targeting the 897 bp hexon gene. The PCR product was subjected to gel electrophoresis, to check 897 bp size amplicon. The PCR protocol was same as that of practiced to screen FAdV in liver samples.

**RESULTS AND DISCUSSION**

Recently, fowl adenoviruses have gradually emerged as the primary pathogen associated with inclusion body hepatits, hydropericadial hepatits syndrome conditions in young broiler chicken causing important economic loss worldwide in recent decades [10]. In India, the occurrence and reemergence of FAdV diseases has been increasing in trend in the recent past and causes major economic loss to the poultry farmers. Clinical signs such as increased mortality, depression, dullness, reduced feed intake, depression, ruffled feathers, changes in posture and, in some flocks watery diarrhoea and leg weakness, along with hepatitis outbreaks in young broilers as well as day old broiler chicks in recent times reported in India [6]. In this study, we screened liver samples collected from 100 flocks of commercial broiler chicken suspected for FAdV infection from eleven different states, including Tamil Nadu and found 40 flocks were positive for the 897 bp hexon gene of FAdV (Fig 1.). Sequencing and phylogenetic analysis revealed all the isolates belonged to FAdV serotype 11 of group D. Similar findings were reported by various researchers [12, 13, 14].

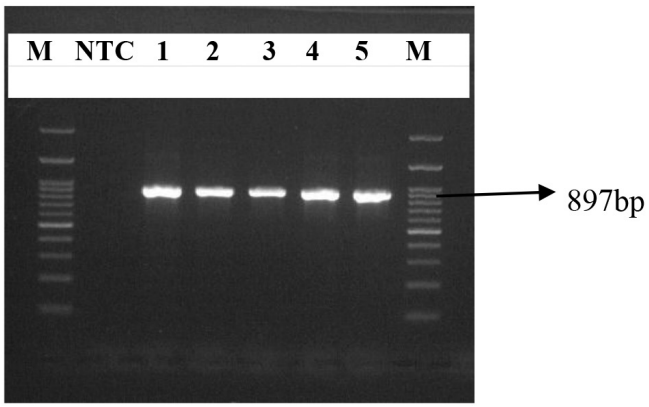


**Fig. 1. Agarose gel electrophoresis showing 897 bp amplified PCR productof hexon gene of field fowl adenoviruses**

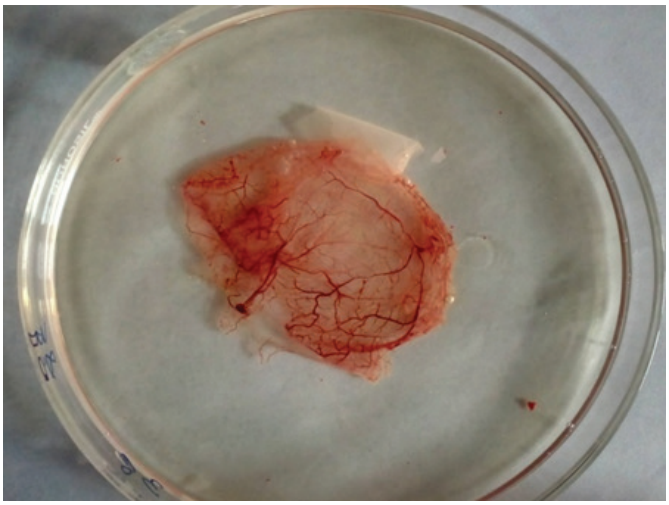

**Fig. 2. Infected CAM and no prominent lesions**

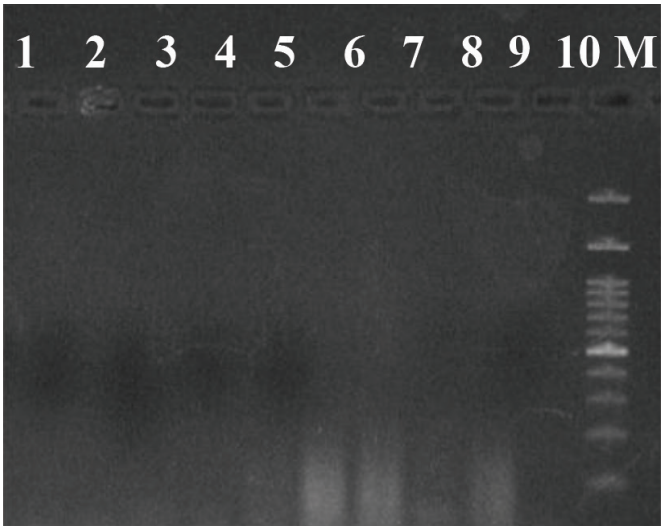


**Fig. 3. Agarose gel electrophoresis showing no amplification.** [M - 100 bp DNA Marker;Lane 1 to 5 - Infected CAM; Lane 6 to 10 - Embryo liver].

Fowl adenoviruses can be isolated from faeces, liver, trachea, gizzard and pharynx but liver is the most common specimen of choice for isolation of virus [15]. In our study, for isolation of field FAdV isolates (FAdV serotype 11), fourteen out of 40 representative FAdV positive liver tissue samples by PCR with no other concurrent infections like CAV, ALV, MDV and REV were directly inoculated and passaged in eleven days old embryonated chicken egg via CAM route and up to five passages were carried out. There is no death of embryo or specific lesions were found on the CAM and embryos throughout the incubation period. The

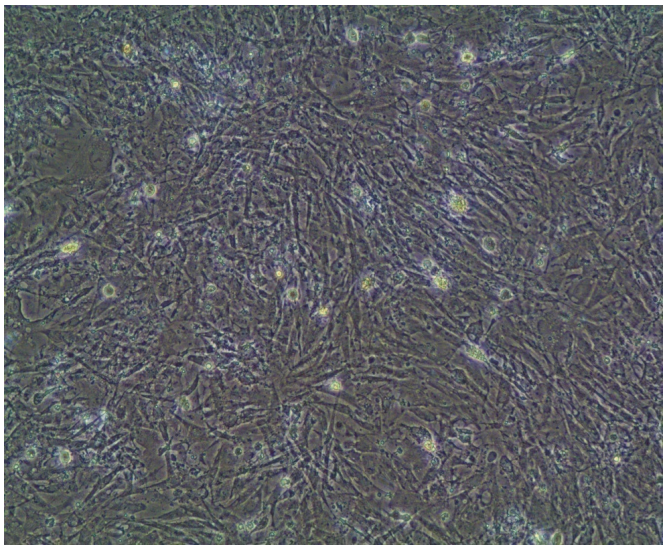

**Fig. 4. Uninfected chicken embryo fibroblast culture 72h (100 X).**

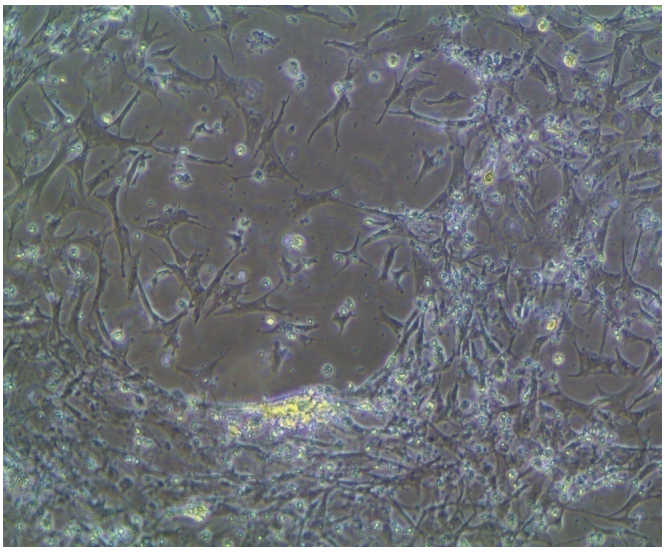

**Fig. 5. Cytopathic effect induced by the field FAdV isolate in CEF at 5th passage at 72 h (100 X).**

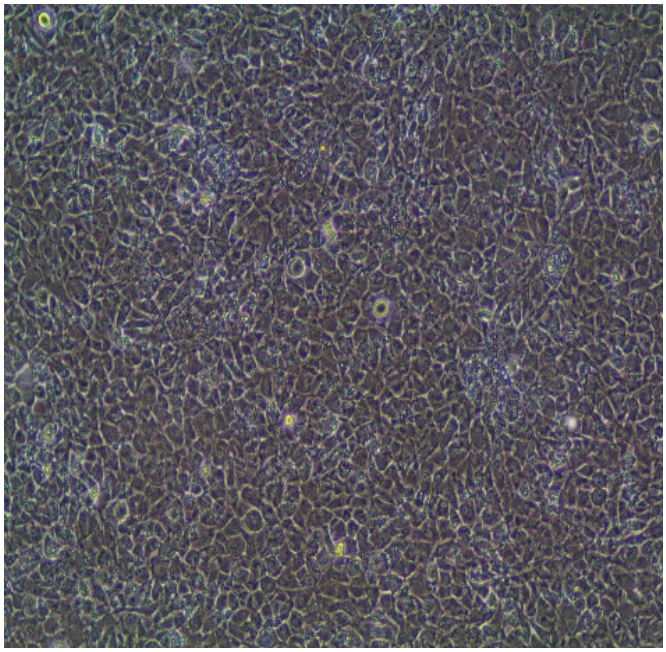

**Fig. 6. Uninfected chicken embryo liver culture at 72h (100 X).**

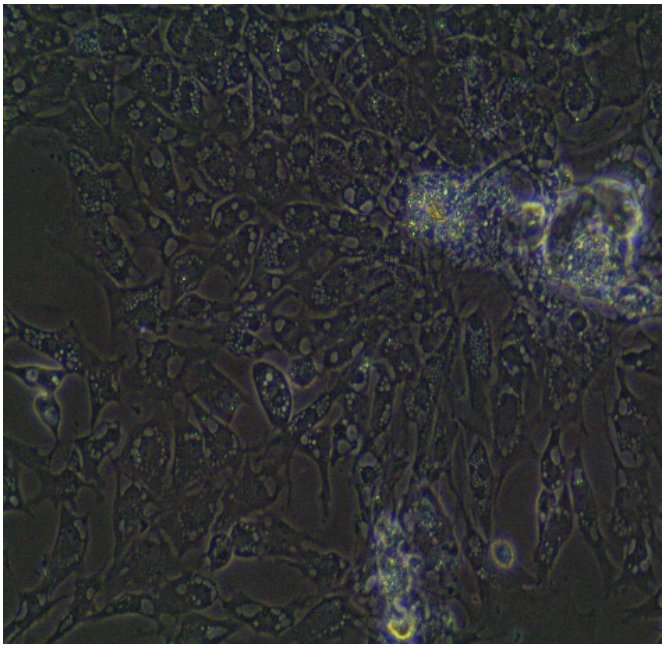

**Fig. 7. Cytopathic effect induced by the field FAdV isolate at 24h in CELi culture of 3rd passage (100 X).**

DNA extracted from CAM and liver tissues up to five passages were subjected for PCR targeting hexon gene of FAdV revealed no amplification (Fig. 2. & 3). In this study, field FAdV isolates could not be isolated using CAM route of embryonated egg inoculation. Chicken embryo liver or kidney cells or embryonated chicken eggs are used for virus isolation but not all FAdVs multiplies in embryonated egg and cause recognizable lesions [5]. Kawamura *et al*. [16] reported that when high virus concentrations were inoculated via CAM all serotypes except serotype 3 produced lesions. Whereas Burke *et al*. [17] made only three isolates in embryonated eggs compared to 45 in cell culture. The probable reason for not adaptation of virus in ECEs via CAM might be due to be very low infectious virus concentration in the samples or difference in virulence of the virus.

For virus isolation in CEF cell culture, nine out of fourteen PCR positive liver tissues produced CPE consisting of cell rounding, clumping and detachment

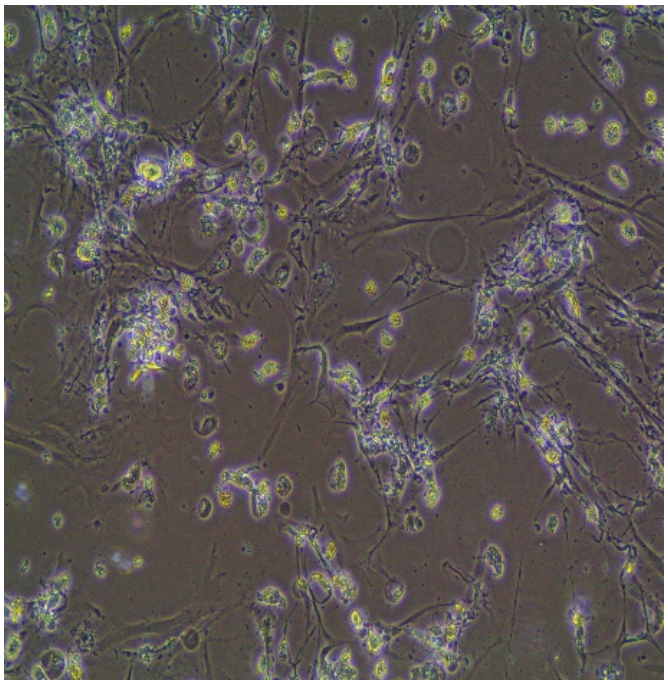

**Fig. 8. Uninfected chicken embryo liver culture at 72h (100 X).**

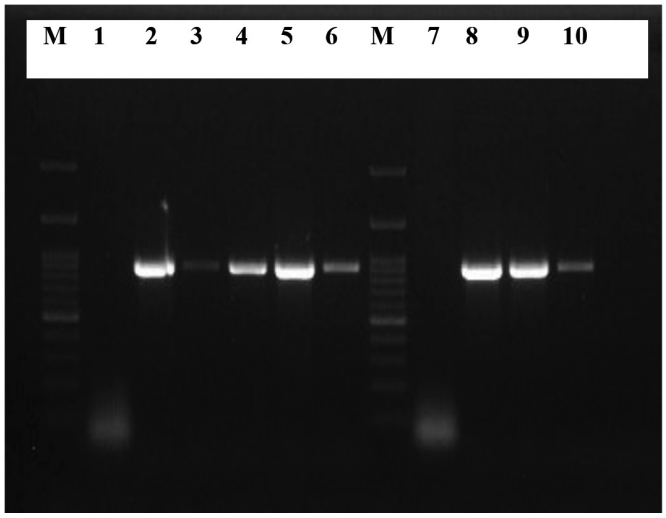


**Fig. 9. 1-CEF uninfected, 2 to 6 : CEF-1st passage to 5th passage.** [M- DNA ladder 7-CELi uninfected, 8 to 10 – CELi - 1st passage to 3rd passage].

and floating of cells at 72 h of 5th passage (Fig. 4 & 5). However, the CPE was observed at fifth passage level and freeze thawed infected cell lysates of all five passages were positive for FAdV targeting hexon gene specific PCR. (Fig. 9). This finding was supported by Miller *et al.* [18] who stated CEF and tracheal organ cultures were less sensitive for isolation of adenovirus than the chick kidney and chick embryo liver cell cultures. In this study, virus replication takes place in all five passages but clear CPE observed at fifth passage level only. Fowl adenovirus isolation using CEF cell culture was studied by many researchers and virus recovered at different passage levels were reported [7, 19].

In chicken embryo liver cells, first two passages CPE consisting of rounding of cells and detachment was observed at 48 to 72 h but in the third passage onwards CPE consisting of presence of vacuole and honeycomb appearance at 24 h post infection followed by cell rounding, clumping, detachment and floating of cells were observed. (Fig. 6, 7, 8). All the fourteen isolates were recovered from liver samples using CELi cells and viruses are well adopted and propagated in liver cell culture. After alternate freezing and thawing of all passage's lysates were found positive for hexon gene of FAdV specific PCR (Fig.7). Therefore, CELi culture is more sensitive and useful for isolation of field FAdV. The results of this study well correlated with the results of Soumyalekshmi *et al.* [20] and Trivedi *et al.* [21].

## CONCLUSION

This study found that fowl adenovirus infection is common in commercial broiler chickens in India and is responsible for their considerable rate of mortality and morbidity. All the 14 field FAdV isolates had belonged to serotype 11 and well adopted and propagated using primary CELi cells with typical CPEs of FAdV infection. Massive destruction of cell monolayer within early period of time was observed in CELi cells as a indicator of fowl adenoviral replication. In this study, field FAdV isolates were not well adopted in CAM route of embryonated chicken eggs and cause recognizable lesions, this might be due to variation in the concentration of virus or difference in virulence at the time of inoculation. For propagation and isolation of the FAdV, CEli cells are suitable culture system when compared to primary CEF cell culture and Embryonated eggs.

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