RESEARCH ARTICLE

Isolation and Histopathological Studies of Fowl Adenovirus from Inclusion Body Hepatitis Suspected Chicken

Babu Naik Kethavath[1*], Srivani Moturi[1], Deepthi Balam[2], Srinivasa Rao Tumati[3], Sudha Rani Chowdary Chitturi[4]

Abstract

Inclusion body hepatitis (IBH) is one of the major economically significant emerging diseases in broilers caused by various serotypes of fowl adenovirus (FAdV). Routine outbreaks were recorded, despite regular vaccination in breeders and young chicks. The aim of the present study was isolation, molecular confirmation and histopathological studies of FAdV associated with IBH. A total of 34 pooled liver samples of IBH suspected broiler, broiler breeder, layer and desi chickens were collected from different districts of Andhra Pradesh. Liver samples were initially screened by PCR and histopathology of liver tissues. Among these, 19 samples were found to be positive for L1 region of partial hexon gene in PCR, of which 5 were sequenced. These samples showed more than 99% sequence homology with serotype FAdV-11 of species D. These FAdV positive and sequenced liver tissues were selected for isolation in SPF embryonated chicken eggs (ECE) and chicken embryo liver (CEL) primary cell cultures. Infected embryos displayed haemorrhagic, shrunken with enlarged, mottled livers with the presence of basophilic intra-nuclear inclusion bodies within hepatocytes, while CEL primary cell culture exhibited characteristic cytopathic effects, including rounding, aggregation, clumping, detachment and floating. The presence of the virus was further identified by PCR and histopathology.

Key words: Cytopathic effects (CPE), Fowl adenovirus (FAdV), Inclusion body hepatitis (IBH), Isolation, Serotype FAdV-11.

Ind J Vet Sci and Biotech (2026): 10.48165/ijvsbt.22.3.11

Introduction

Inclusion body hepatitis (IBH) is a rapidly emerging and acute disease in chickens, characterized by severe anaemia and high mortality, caused by fowl adenoviruses (FAdVs) (Shinde *et al*., 2020). Despite routine vaccination, outbreaks persist in broiler chickens due to the presence of multiple serotypes with limited cross-protection. FAdVs are non-enveloped viruses, measuring 70-80 nm in diameter, with a single linear double-stranded DNA (dsDNA) genome encased within an icosahedral structure composed of 252 capsomers. According to the International Committee on Taxonomy of Viruses (ICTV), FAdVs are classified within the genus *Aviadenovirus* and belong to the family *Adenoviridae*.

According to Rahimi & Haghighi (2015), IBH was first identified in USA in 1963 by Helmboldt and Frazier. In Asia, IBH was initially reported in Angara Goth near Karachi, Pakistan (Rahimi *et al*., 2015; Gulhane *et al*., 2016). IBH typically affects broiler chickens at the age of 3 to 7 weeks and causes mortality rates of 10-30% (Sharma *et al*., 2018), though this can rise to as high as 80% depending on the virulence of the virus, the immune status of the flock and the presence of secondary infections (Panigrahi *et al*., 2016). Based on restriction enzyme digestion patterns, FAdVs are categorized into five molecular species (FAdV-A to FAdV-E) under group-1, and 12 serotypes (FAdV-1 to 8a and FAdV-8b to 11) based on cross-neutralization assays (Abghour *et al*., 2019; Sohaimi *et al*., 2019; Kumar *et al*., 2022; Shankar *et al*., 2022). In Indian broilers, serotypes 2, 8a, 8b and 11, associated with species D and E, have been identified as the primary agents causing IBH (Shankar *et al*., 2022).

[1]Department of Veterinary Microbiology, NTR College of Veterinary Science, Sri Venkateswara Veterinary University, Gannavaram-522102, Andhra Pradesh, India

[2]Department of Veterinary Microbiology, College of Veterinary Science, Sri Venkateswara Veterinary University, Garividi-535101, Andhra Pradesh, India

[3]Department of Veterinary Public Health & Epidemiology, NTR College of Veterinary Science, Sri Venkateswara Veterinary University, Gannavaram-521102, Andhra Pradesh, India

[4]Department of Veterinary Pathology, NTR College of Veterinary Science, Sri Venkateswara Veterinary University, Gannavaram-522102, Andhra Pradesh, India

Corresponding Author: Dr. Babu Naik K., MVSc Scholar, Department of Veterinary Microbiology, NTR College of Veterinary Science, Sri Venkateswara Veterinary University, Gannavaram-522102, Andhra Pradesh, India. e-mail: kbabunaik97@gmail.com

How to cite this article: Babu Naik, K., Srivani M., Deepthi, B., Srinivasa Rao, T., & Sudha Rani Chowdary, CH. (2026). Isolation and Histopathological Studies of Fowl Adenovirus from Inclusion Body Hepatitis Suspected Chicken. Ind J Vet Sci and Biotech, 22(3), 57-61.

Source of support: Nil

Conflict of interest: None

Submitted 10/12/2025 **Accepted** 12/01/2026 **Published** 10/05/2026

FAdV can be isolated from various samples, including faeces, pharynx, trachea and affected organs such as the liver and gizzard. Among these, liver is considered as the specimen of choice for virus isolation due to its reliability (Chitradevi *et al*., 2021). For isolation of FAdV, embryonated chicken eggs (ECEs) as well as chicken embryo liver or kidney primary cells were commonly employed. Numerous studies have demonstrated that chicken embryo liver cells are the most effective system for isolation of FAdV (Trivedi *et al*., 2018; Sohaimi *et al*., 2019). Histological investigations play a key role in laboratory diagnosis, with the identification of intranuclear inclusion bodies in hepatocytes serving as a hallmark feature. The present study was designed to isolate and characterize the FAdV in the current IBH outbreaks in Andhra Pradesh broiler chickens by PCR and histopathological investigation.

Materials and Methods

Sample Collection and Processing

Liver tissue samples (3-4) from each flock were collected and pooled from 34 chicken flocks suspected of having IBH across various districts of Andhra Pradesh, *viz,* Chittoor, Prakasam, Palnadu, Guntur, NTR, Krishna, Eluru, Bapatla, East Godavari and West Godavari during September 2023 to October 2024. These included 23 samples from commercial broiler flocks, 4 from broiler breeder flocks, 5 from layer flocks and 2 from desi flocks (Table 1). All the liver samples were collected in sterile phosphate-buffered saline (PBS) with a pH of 7.0 to 7.4, containing antibiotics (100 units of penicillin G, 100 µg of streptomycin and 0.25 µg of amphotericin B/mL). The samples were then carried to the laboratory in an ice box with ice packs and stored at -80°C until processing.

Table 1: District wise sample collection details in Andhra Pradesh

District	Commercial broiler flocks	Broiler breeder flocks	Layer flocks	Desi flocks
Chittoor	2	0	0	0
Prakasam	2	1	0	0
Palnadu	3	0	1	0
Guntur	1	1	0	0
NTR	8	0	1	0
Eluru	3	0	1	1
Krishna	2	0	0	0
East Godavari	0	2	0	0
West Godavari	0	0	2	0
Bapatla	2	0	0	1
Total	**23**	**4**	**5**	**2**

Molecular Detection by PCR

DNA was extracted from tissue homogenate by using the HiPurA® Mammalian Genomic DNA Purification Kit (Hi-Media), following the protocol provided by the manufacturer. Gene-specific primers F: 5'-CAARTTCAGRCAGACGGT-3' and R: 5'-TAGTGATGMCGSGACATCAT-3' were used to amplify L1 region of partial hexon gene (Meulemans *et al*., 2001).

Materials and Reagents for Isolation of FAdV

The materials and reagents used in the isolation of the virus were Dulbecco's Modified Eagle's Medium (DMEM): High glucose (4500 mg/L) with L-glutamine (Sigma, Cat# SLBW4140). Sodium bicarbonate: 3.7 g/L (Hi-Media). Fetal bovine serum (FBS) (Gibco™, Cat# 1009130). Growth medium (10% FBS) and maintenance medium (2% FBS). Trypsin-Versene Solution: 0.17% trypsin and 0.14% versine (Hi-Media). Phosphate Buffered Saline (PBS): pH 7.4. Embryonated Chicken Eggs (ECEs): 12-14 days old (Srinivasa Hatcheries Ltd., Andhra Pradesh). Tissue culture flasks and tissue culture plates (Tarsons).

Isolation of FAdV from ECEs

To isolate the FAdV virus, PCR-positive pooled liver tissues (3-4) were homogenized to prepare a 20% w/v suspension using sterile PBS (pH 7.2). The suspension was centrifuged at 805x g for 10 min at 4°C and the supernatant was treated with chloroform in a 4:1 ratio. This mixture was centrifuged again at 805x g for 15 min at 4°C and the resulting supernatant was filtered through a 0.22 µm membrane filter with 1% antibiotic added. The prepared inoculum was then inoculated (0.1 mL) into allantoic sac route in 10-11 day old embryonated chicken eggs (ECEs) using a sterile syringe at a 45º angle. A negative control using sterile PBS was also maintained. The eggs were sealed with sterile wax and incubated at 37° C for 6 days, with daily candling to monitor viability and positional changes. After incubation, the embryos were chilled at 4 ºC for 8-12 h.

The embryos were then swabbed with 70% alcohol and the shells were carefully opened. The chorioallantoic membrane (CAM) was peeled back and allantoic fluid was collected for DNA extraction. The embryos were washed in sterile PBS, ventrally transected and the livers were aseptically dissected. The livers were washed twice with PBS for further analysis.

Isolation of FAdV from CEL Primary Cell Culture

CEL primary cell cultures were prepared using a modified method of Adair *et al*. (1979). Livers from 12-14 day old embryos were aseptically collected, washed with PBS (pH 7.4), finely minced and trypsinized with a sterile trypsin-versene solution (0.17% trypsin and 0.14% versene) at 37°C to dissociate the cells. The suspension was filtered through muslin cloth, centrifuged at 358 x g for 10 min and the cell pellet was washed with DMEM containing 10% NBCS. The pellet was resuspended in DMEM with 10% FBS, adjusted to a concentration of 3×10^6 cells/mL and distributed into 25 cm^2 flasks. After incubation at 37°C in a 5% CO_2 incubator for 48-72 h, a uniform monolayer of CEL cells was established. This monolayer was then inoculated with virus inoculum, 200 µL of stock virus was diluted with 1800 µL of 2% maintenance medium. Monolayers were washed with serum-free DMEM, inoculated with 2 mL of virus inoculum per flask and incubated at 37°C in a 5% CO_2 incubator for 60 min to allow adsorption. After decanting the inoculum, maintenance medium with 2% FBS was added and the cultures were incubated while monitoring for cytopathic effects (CPE) for up to 4 days. Monolayers showing more than 80% CPE were harvested, subjected to three freeze-thaw cycles and clarified by centrifugation at 850 x g for 10 min at 4°C. The supernatant was collected and stored at -80°C.

Histopathology

The histopathological slide preparation of field and CEL tissues were performed at the Department of Pathology, Dr. Pinnamaneni Siddhartha Institute of Medical Sciences and Research Foundation, Chinna Avutapalli, Krishna district, Andhra Pradesh. The prepared slides were then analysed at the Department of Veterinary Pathology, NTR College of Veterinary Science, Gannavaram, Krishna district, Andhra Pradesh.

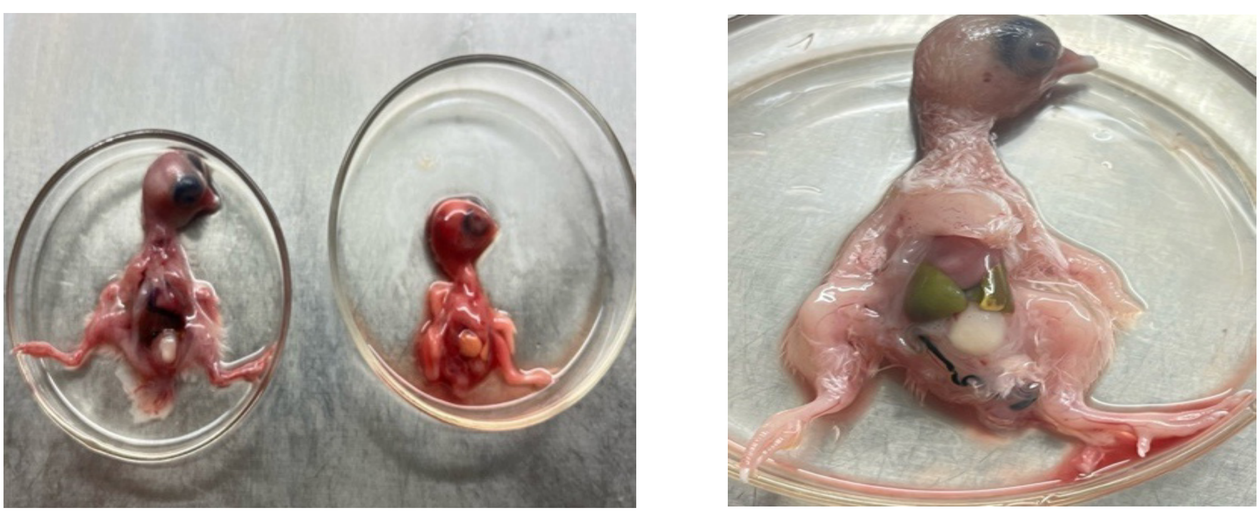
Results and Discussion

Embryos inoculated with liver homogenates of infected chickens were incubated at 37°C for six days. No mortality was observed during this period. Hence all the embryos were chilled for further processing. Virus inoculated embryos were shrunken, haemorrhagic, and exhibited enlarged liver with yellow to reddish foci or diffuse greenish discoloration (Fig. 1), which were consistent with the findings of Agbhour *et al*. (2019).

Avian origin cells such as primary CEL cell cultures are superior for FAdV adaptation (Sohaimi *et al*., 2019; Chitradevi *et al*., 2021). In this study FAdV isolate was successfully adopted and propagated for up to three passages in primary CEL cells with typical CPE of FAdV infection. The virus caused cells rounding, aggregation, clumping and detachment of sheet and floating of CEL cells. These changes are consistent with the findings of Gulhane *et al*. (2016) and Chitradevi *et al*. (2021). The CPE of adenovirus may be very characteristic, with rounding and clumping of affected cells into regular clusters resembling "bunches of grapes" (Sawale *et al*., 2012). This study observed CPE in 48 h of PI at the first passage, which is in agreement with Kumar *et al*. (2013), who observed rounding and degeneration of cells from first passage. Contrast to the present findings, Chitradevi *et al*. (2021) observed CPE during second passage. CPE formation with massive destruction of monolayer culture in this study was an indication of viral replication. This is in accordance with the changes observed by Sohaimi *et al*. (2019) (Fig. 2).

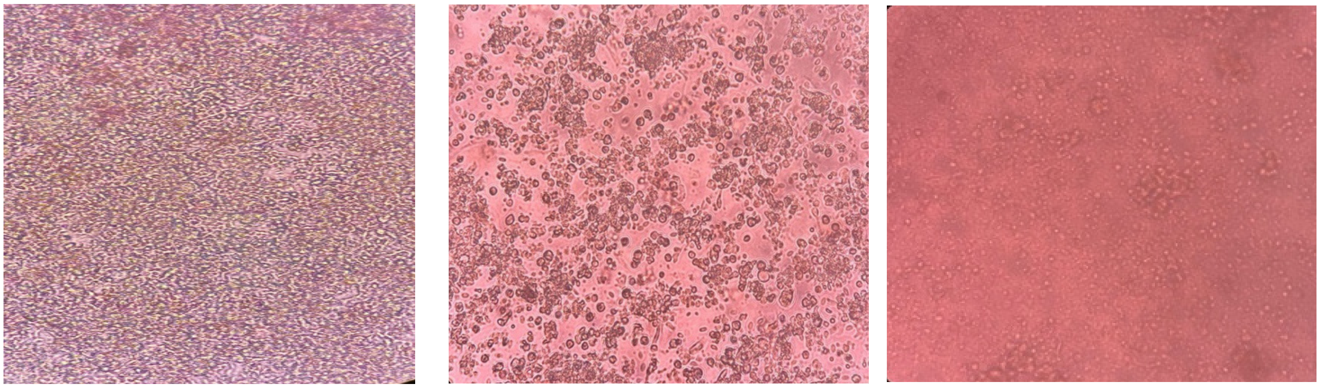
Extracted DNA from ECEs and CEL primary cell cultures were subjected to PCR by targeting hexon gene and obtained 897 bp band in amplified samples (Fig. 3). This confirms the FAdV replication in ECEs and CEL primary cell cultures. However, no amplification was detected in the uninfected cell cultures or chicken embryo liver cells (negative control).

Histopathological analysis of liver tissues from chickens affected by IBH revealed significant hepatic damage characterized by extensive multifocal necrosis and random disruption of hepatocellular architecture. Basophilic intranuclear inclusion bodies, a hallmark of FAdV infection are observed in hepatocytes, both in field samples (Fig. 4) and virus-inoculated embryos (Fig. 5). These findings were confirmed using hematoxylin and eosin (H&E) staining, aligned with previous reports by Gulhane *et al*. (2016), Agbhour *et al*. (2019), Shiyamala *et al*. (2020), and Chitradevi *et al*. (2021). These inclusion bodies, formed by the accumulation of viral proteins and DNA during active viral replication, are pathognomonic of FAdV infection. These lesions highlight the profound impact of FAdV on liver structure and cellular integrity in affected chickens.



A B

Fig. 1: A) Isolation of FAdV in ECEs (6 day PI). Shrunken infected embryo compared to the control embryo, **B)** Enlarged liver with a diffuse greenish discoloration in FAdV inoculated embryo.



A B C

Fig. 2: A) Uninfected monolayer of CEL primary cell culture (200X), **B)** Rounding and clumping of cells after 48 hours of PI (200X), **C)** Detachment of sheet after 72 hours of PI (200X)

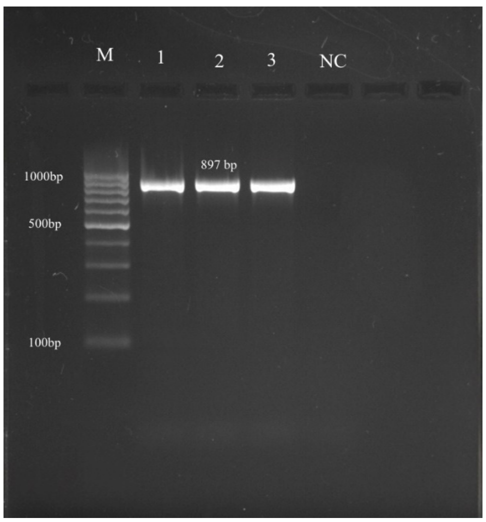
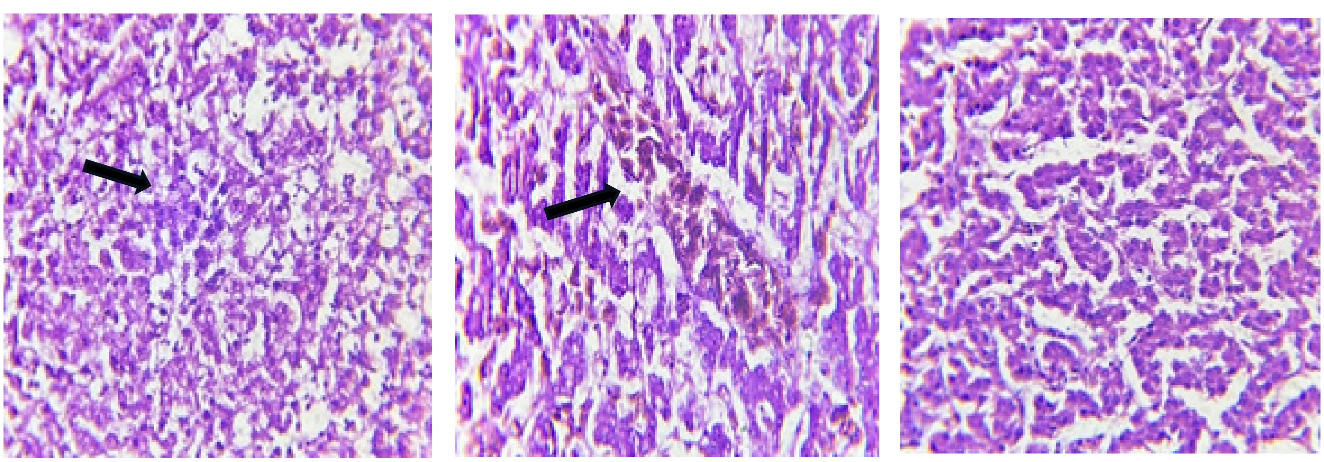


Fig. 3: Gel photograph of PCR assay targeting partial hexon gene (897bp). Lane M: DNA ladder (100bp), Lane 1: Positive control, Lane 2: FAdV isolate from cell culture, Lane 3: FAdV isolate from ECE liver. Lane NC: Negative control.

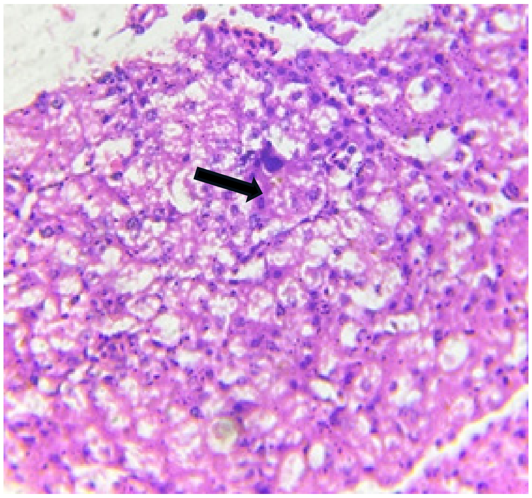
Conclusion

This study provides valuable insights into the virological and pathological features of fowl adenovirus (FAdV) associated with inclusion body hepatitis (IBH) in chickens. Experimental infection of embryonated chicken eggs (ECEs) and propagation in primary chicken embryo liver (CEL) cell cultures demonstrated characteristic gross and microscopic lesions, confirming FAdV replication. Cytopathic effects (CPE) such as cell rounding, aggregation and clumping were observed, alongside PCR confirmation of the virus. Histopathological analysis revealed severe hepatic damage, including necrosis and basophilic intranuclear inclusion bodies, highlighting the virus's impact on liver integrity. The findings underscore the value of histopathology, cell cultures and molecular techniques in diagnosing and understanding FAdV infections, aiding in the development of effective control strategies for IBH in poultry.



A B C

Fig. 4: A) FAdV infected chicken liver showing basophilic intranuclear inclusion bodies (H&E×400), **B)** Mild to moderate congestion in FAdV infected chicken liver, **C)** Healthy chicken liver showing normal hepatocytes (H&E×400).



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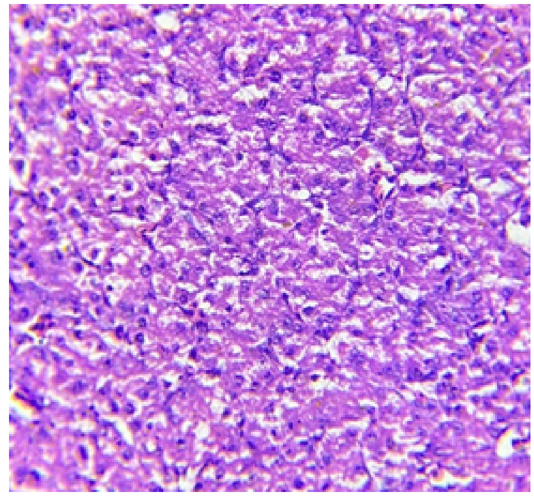

B

Fig. 5: A) FAdV infected embryo liver showing basophilic intranuclear inclusion bodies (H&E×400), B) Image of control embryo liver showing normal hepatocytes (H&E×400).

Acknowledgements

The authors acknowledge NTR College of Veterinary Science, Gannavaram and Sri Venkateswara Veterinary University for providing the opportunity and required facilities to undertake research work and special thanks to the individuals and farm owners who helped in the procurement of clinical samples.

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