

Molecular detection of fowl adenovirus (FAdV) from inclusion body hepatitis (IBH) suspected chicken in Andhra Pradesh

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Abstract

Inclusion body hepatitis (IBH) is an emerging disease of economic concern in chickens, primarily caused by various serotypes of fowl adenovirus (FAdV). Despite regular vaccinations, outbreaks are being reported in chicken flocks of Andhra Pradesh due to the presence of multiple serotypes, with limited cross-protection. Hence, the present study was aimed to confirm the presence of IBH in commercial and desi chickens, and to identify the circulating FAdV serotypes. Liver samples were obtained from 34 commercial and desi chicken flocks with suspected IBH from ten different districts of Andhra Pradesh. Necropsy findings revealed hydropericardium, hepatomegaly, petechial haemorrhages on the liver, yellowish discoloration of subcutaneous and abdominal fat, as well as kidney oedema and paleness. Molecular detection of FAdV in the samples was carried out using PCR primers by targeting the hypervariable region of the L1 loop of the hexon gene yielding an amplicon with a size of 897 bp. Out of the 34 flocks tested, 19 flocks (55.88%) were found to be positive. To identify the circulating serotypes, five PCR products from different districts were sequenced. The phylogenetic tree was constructed by retrieving FAdV reference sequences from the GenBank database and all exhibited 99-100% similarity with field isolates. This study concluded that FAdV serotype 11 was the prevalent serotype in IBH outbreaks in chicken flocks of Andhra Pradesh.

Keywords: Broiler flocks, Fowl adenovirus (FAdV), Inclusion body hepatitis (IBH), Sequencing, Serotype 11

Highlights

- IBH is a disease of economic significance in chickens, mainly due to different serotypes of FAdV infection
- Necropsy revealed hydropericardium, hepatomegaly with petechial haemorrhages and kidney oedema
- Various FAdV serotypes offer limited cross-protection leading to frequent outbreaks
- PCR targeting hexon gene of capsid protein confirmed IBH in 19 out of 34 chicken flocks in Andhra Pradesh
- FAdV serotype 11 was the prevalent serotype associated with IBH in chicken flocks

INTRODUCTION

Indian poultry industry is one of the fastest growing segments of the agricultural sector in India. The poultry industry is threatened by various infections and non-infectious diseases, which are responsible for major economic losses. One such emerging disease that causes considerable mortality in broilers is inclusion body hepatitis (IBH), induced by fowl adenoviruses (FAdVs), which are a diverse group of viral pathogens causing a range of diseases in poultry. The virus is non-enveloped, measuring 70-80 nm in diameter, with a linear double-stranded DNA (dsDNA) enclosed in an icosahedral capsid structure, consisting of 252 capsomers. As per the current international committee on taxonomy of viruses (ICTV) classification, the virus is classified under the genus *Aviadenovirus* belongs to the family *Adenoviridae*. Based on the restriction

enzyme digestion (RFLP) pattern, there are 5 molecular species in group-1 viz., FAdV-A to FAdV-E and there are 12 serotypes from FAdV 1 to 8a and FAdV 8b to 11 based on the cross-neutralization assay (Abghour *et al.*, 2019; Sohaimi *et al.*, 2019; Kumar *et al.*, 2022; Shankar *et al.*, 2022). Fowl adenovirus serotypes 2, 3, 9 and 11 of species D and 6, 7, 8a and 8b serotypes of species E are main cause of IBH. However, serotypes 2, 8a, 8b and 11 of two species (D and E) are being the cause of IBH in Indian broilers (Shankar *et al.*, 2022).

IBH was recognized for the first time in the USA by Helmboldt and Frazier in 1963. Since then, the disease has been reported in many countries of the world. In Asia it was first identified in Angara Goth near Karachi in Pakistan (Rahimi and Haghighi, 2015; Gulhane *et al.*, 2016). In the early eighties, the occurrence of IBH with low mortality was recorded in India and other

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parts of the world. It normally affects broiler chickens at the age of 3 to 7 weeks (Sharma *et al.*, 2018), with 10-30% mortality (Chitradevi *et al.*, 2021; Mohamed and El-Sabagh, 2023). Mortality may reach up to 80% depending on the virus pathogenicity, chicks immune status and the presence of concurrent secondary infections (Panigrahi *et al.*, 2016). Previously, FAdV was reported to be associated with immunosuppressive viruses like infectious bursal disease virus (IBDV) and chicken infectious anaemia virus (CIAV) as secondary infections (Kumar *et al.*, 2022). Nonetheless, it is now becoming apparent as the primary infection in chicken, resulting in significant financial losses (Gulhane *et al.*, 2016). However, IBH was also reported in other avian species, including turkeys, pigeons, geese, psittacine birds. The symptoms in affected birds includes reduced feed intake, ruffled feathers, poor growth, lethargy and inability to walk. The prominent necropsy findings were enlarged liver with pale, friable and mottled appearance with necrotic foci, along with hydropericardium characterized by clear, straw-coloured fluid. Ecchymotic haemorrhages may also be seen in liver and less consistently in thigh and breast muscles. Kidneys often appear enlarged and congested. In most cases, lesions were observed in the liver (Sawale *et al.*, 2012; Rahimi *et al.*, 2015; Chitradevi *et al.*, 2021).

Diagnosis of IBH can be carried out through a combination of conventional, histological and molecular methods. Observation of gross and histopathological changes is a traditional approach, while advanced diagnostic tools such as polymerase chain reaction (PCR) and sequencing have facilitated

rapid and specific detection of FAdV (Raue and Hess, 1998; Meulemans *et al.*, 2001; Abghour *et al.*, 2019). Molecular serotyping, based on hexon gene coding sequences, is currently employed for FAdV characterization, which replaced the traditional cross-neutralization assays and restriction fragment length polymorphism (RFLP) due to their complexity and labour-intensive nature.

In spite of regular vaccination, routine outbreaks are occurring in broiler farms of Andhra Pradesh due to multiple serotypes with less cross-protection (Chitradevi *et al.*, 2021). Therefore, it is essential to comprehend circulating serotypes for effective management and prevention of the disease. Since there is a paucity of information on the circulating serotype in this geographic area, the current study was undertaken to detect fowl adenovirus associated with IBH from suspected chicken and to identify circulating FAdV serotypes based on phylogenetic studies.

MATERIALS AND METHODS

Sample collection: Based on clinical signs and post mortem lesions in 34 chicken flocks suspected of having IBH, liver tissue samples (3-4) from each flock were collected from across ten districts of Andhra Pradesh, *viz.*, Chittoor, Prakasam, Palnadu, Guntur, NTR, Krishna, Eluru, Bapatla, East Godavari and West Godavari. These samples were obtained from outbreaks that occurred in these districts of Andhra Pradesh between September 2023 and October 2024. 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 processed. The collected samples were listed in the Table 1.

Genomic DNA extraction: Liver samples collected from clinically affected chickens were ground using a sterile mortar and pestle to ensure thorough homogenization. A 20% suspension was prepared with PBS, followed by centrifugation at 3000 rpm for 10 minutes. The supernatant was then collected for DNA extraction. DNA extraction from pooled liver samples were performed by using the HiPurA® Mammalian Genomic DNA

Table 1. District wise sample collection details in Andhra Pradesh

S. No.	District	Commercial broiler flocks (2-6 wks)	Broiler breeder flocks (40-42 wks)	Layer flocks (0-86 wks)	Desi flocks (2-7 wks)
1.	Chittoor	2	0	0	0
2.	Prakasam	2	1	0	0
3.	Palnadu	3	0	1	0
4.	Guntur	1	1	0	0
5.	NTR	8	0	1	0
6.	Eluru	3	0	1	1
7.	Krishna	2	0	0	0
8.	East Godavari	0	2	0	0
9.	West Godavari	0	0	2	0
10.	Bapatla	2	0	0	1
Total		23	4	5	2

Purification Kit (HiMedia, India).

Molecular detection of FAdV by PCR: FAdV was detected with PCR by using partial hexon gene-specific primers F: 5'-CAARTTCAGRCAGACGGT-3' and R: 5'-TAGTGATGMC GSGACATCAT-3' (Meulemans *et al.*, 2001). DNA concentration and purity were measured using a Nanodrop 200C Spectrophotometer (Thermo Scientific, USA). DNA purity is commonly assessed by measuring the OD ratio at 260 nm and 280 nm. Ideally, the acceptable A260/A280 ratio should fall within the range of 1.6 to 1.8.

The reaction was set up in a total volume of 20 μ L, which included 10 μ L of Emerald Green Master Mix (Takara Bio), 1 μ L each of forward and reverse primers

(20 picomoles), 2 μ L of template DNA (100 ng/ μ L) and 6 μ L of nuclease-free water. The amplification process began with an initial denaturation at 95°C for 10 minutes, followed by 35 cycles consisting of denaturation at 94°C for 1 minute, annealing at 62°C for 30 seconds and extension at 72°C for 50 seconds. After completing the cycles, a final extension was performed at 72°C for 10 minutes. The amplified products were analysed using agarose gel electrophoresis in a 1.5% agarose gel and stained with ethidium bromide to check the presence of an 897 bp amplicon by the Gel Doc™ system (Bio-Rad, USA).

Sequencing and phylogenetic analysis: The nucleotide sequence of amplified PCR product of hexon gene was sequenced at M/s IRA Biotech India Pvt. Ltd, Hyderabad, Telangana, India. The obtained sequences were edited for errors and the forward and reverse sequences of each sample were merged into a single sequence using Codon Code Aligner, Version 11.0.1 (a sequence assembly and alignment software). Homology searches were then performed using the NCBI BLAST program (Nucleotide basic local alignment search tool). Reference sequences of FAdV viruses were retrieved from the GenBank database for comparison with the field sequences. Phylogenetic analysis was carried out with the bootstrap values of 1000 replicates of the original data. Multiple sequence alignments were carried out using Clustal W in MEGA version XI (Tamura *et al.*, 2021).

Table 2. Molecular confirmation of fowl adenovirus in commercial and desi chicken by PCR.

S. No	Source of sample examined	No. of samples examined	No. of PCR positive samples for targeting hexon gene (%)
1.	Broiler	23	16 (69.56%)
2.	Layer	5	0
3.	Broiler Breeder	4	2 (50%)
4.	Desi fowl	2	1 (50%)
Total		34	19 (55.88%)

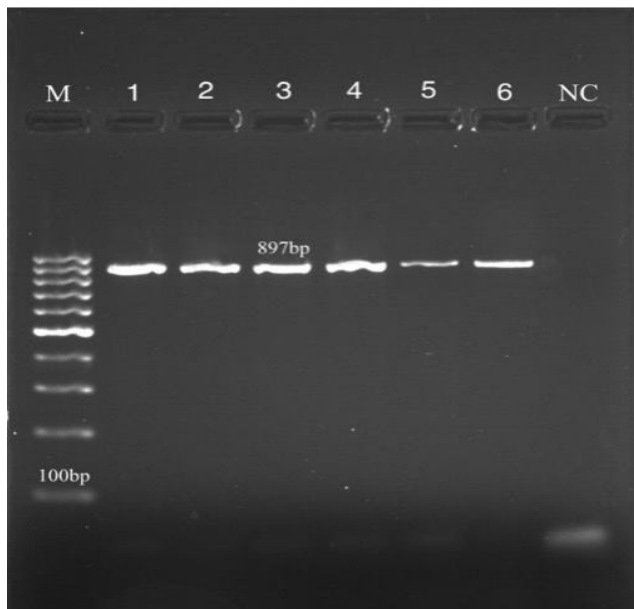


Fig. 1. Gel electrophoresis of PCR assay targeting hexon gene (897bp). Lane M: DNA ladder (100bp), Lane 1: Positive control, Lane 2-6: PCR products of field samples, Lane NC: Negative control.

RESULTS

Molecular detection of FAdV using PCR from liver samples of commercial and desi chickens revealed that, 19 out of 34 flocks were tested positive, with an amplification product of 897 bp, while no amplification occurred in the negative control (Fig. 1). The results revealed that 16 out of 23 broiler flocks (69.56%), 2 out of 4 broiler breeder flocks (50%) and 1 out of 2 desi fowls (50%) were also positive (Table 2), while all the 5-layer flocks were tested negative.

The primary necropsy findings observed were an enlarged, pale, friable liver with pinpoint necrotic foci and small petechial haemorrhages to ecchymosis. Some flocks of commercial broilers exhibited severe kidney enlargement and congestion, yellowish discoloration of subcutaneous and abdominal fat, petechial haemorrhages on the liver with swollen and pale kidneys and in certain flocks, accumulation of 1-2 mL straw coloured fluid in the pericardial sac was also noticed (Fig. 2).

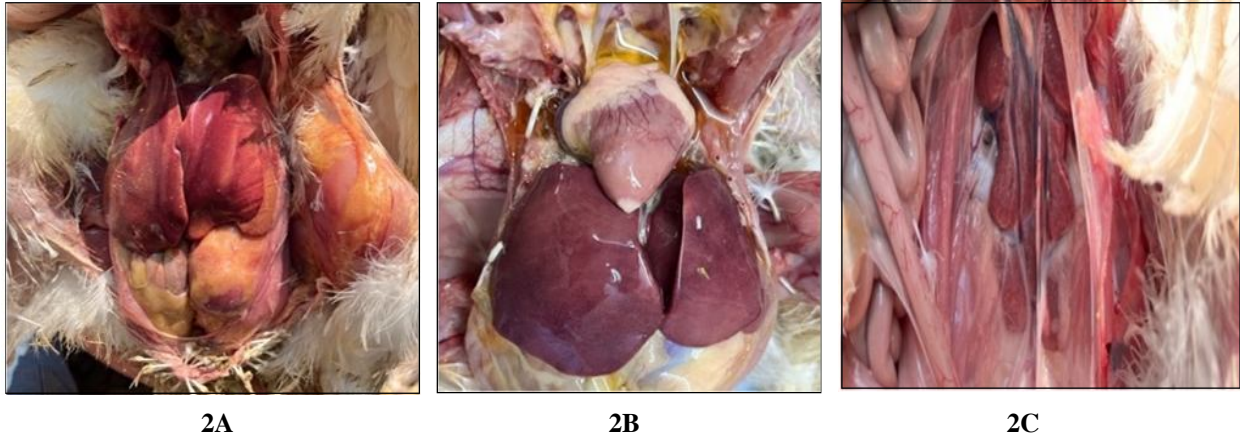


Fig. 2 (A-C). Necropsy findings in inclusion body hepatitis infected broiler chicken. A) Enlarged pale, friable and yellow coloured liver and fatty tissues. B) Straw coloured fluid around the heart. C) Swollen and congested kidneys.

Based on the sample collection data, it was noticed that a seasonal trend exists in flocks affected with FAdV associated with IBH. The highest number of outbreaks occurred during the winter season (December to February) with a prevalence of 63.20%, followed by the monsoon season 26.30% (June to November) and the lowest number of outbreaks was recorded during the summer season 10.50% (March to May) (Fig. 3).

Higher prevalence of IBH (47.05%) was observed in broilers of 4-6 weeks of age, while broiler breeder flocks of around 40 weeks exhibited only 5.88%. However, in desi fowls, a lower prevalence of 2.94% was observed in the 2-7 weeks age group (Table 3), while no outbreak of IBH was recorded in layer flocks.

Nucleotide sequencing and phylogenetic analysis

Sequencing: To determine the origin and genetic relatedness of the FAdV strains from Andhra Pradesh, India, with those circulating in other countries, the coding region of the partial hexon gene from five field strains (FAdV CTR, FAdV NTR, FAdV PLD, FAdV PKS and FAdV GNT) was sequenced. The sequences were submitted to GenBank and the accession numbers were PQ466431, PQ466432, PQ466433, PQ466434 and PQ466435, respectively. The sequencing results of five broiler flocks revealed that all the field sequences showed nearly 99% similarity with local strains on NCBI BLAST analysis obtained from GenBank.

Phylogenetic analysis: A phylogenetic tree (Fig. 4) was constructed using nucleotide sequences of the L1 region of the hexon gene from five selected samples derived from broiler farms across various regions of

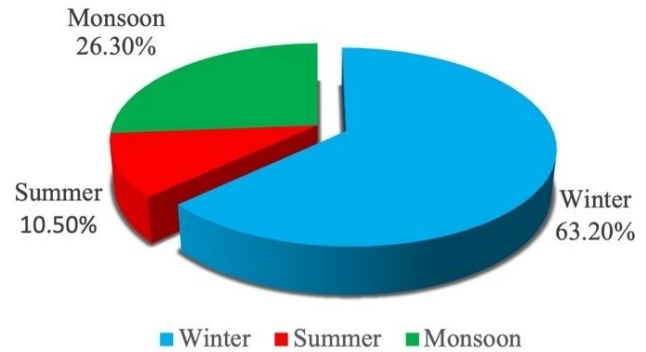


Fig. 3. Pie chart showing season wise prevalence of FAdV associated with IBH

Table 3. Age wise prevalence of fowl adenovirus associated with inclusion body hepatitis

Type of bird	Age in weeks	Positive flocks	Total flocks	Prevalence (%)
Broiler	2-4	0	34	0%
	4-6	16		47.05%
Boiler	40	2		5.88%
Breeder	42	0		0%
Layer	0-86	0		0%
Desi	2-7	1		2.94%

Andhra Pradesh, including Chittoor (FAdV CTR), NTR (FAdV NTR), Palnadu (FAdV PLD), Prakasam (FAdV PKS) and Guntur (FAdV GNT) along with ten sequences of FAdV strains available in GenBank. The phylogenetic analysis showed that the five field sequences clustered closely with FAdV-11 from Brazil, Canada, USA and different states of India, including Telangana, Tamil Nadu and Rajasthan. Whereas

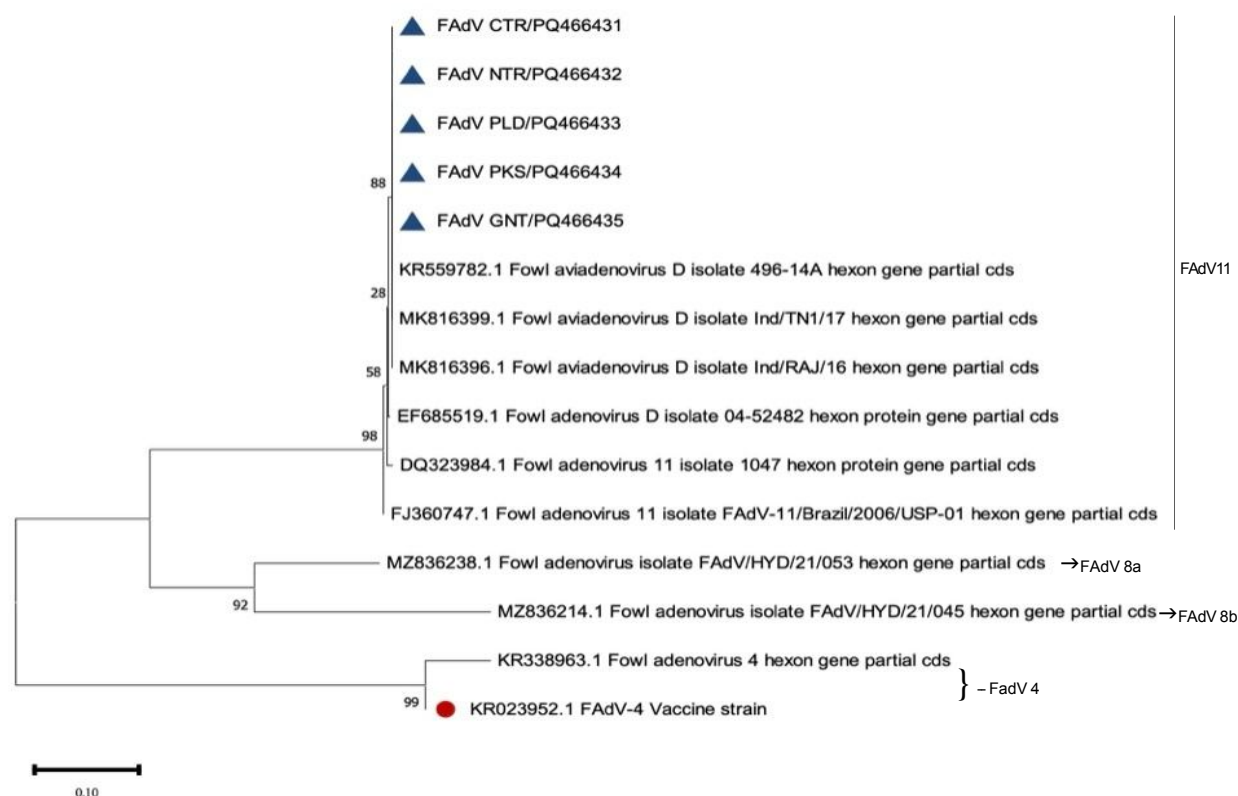


Fig. 4. Phylogenetic tree based on nucleotide sequences of partial hexon gene of FAdV field sequences and sequences from Genbank. Field sequences (▲) and vaccine sequence (●)

FAdV-4, FAdV-8a and FAdV-8b strains formed distinct and separate clusters, indicating genetic divergence.

Based on the phylogenetic grouping of the hexon gene sequences with available GenBank data, the field sequences were confirmed to be FAdV-11 of species D.

Multiple sequence alignment

A multiple sequence alignment was performed using Clustal W, comparing the hexon gene nucleotide sequences of selected samples (PQ466431, PQ466432, PQ466433, PQ466434 and PQ466435) with FAdV sequences of different regions of India, including Telangana (HYD/ KR559782.1), Tamil Nadu (TN/ MK816399.1) and Rajasthan (RAJ/MK816396.1) obtained from NCBI-GenBank revealed 100% nucleotide homology. While with Canada (EF685519.1), USA (DQ323984.1) and Brazil (FJ360747.1) sequences, they exhibited around 99 % nucleotide homology.

DISCUSSION

Inclusion body hepatitis (IBH) has been recognized globally as an emerging disease associated with fowl

adenovirus (FAdV) infection, with increasing reports of sporadic outbreaks causing substantial economic losses to the broiler industry. This study observed PCR detection targeting the L1 region of the hexon gene of FAdV capsid protein produced a single, specific band of 897 bp in 19 flocks originated from livers of broiler, broiler breeder and desi fowls, which were consistent with the findings of Shiyamala *et al.* (2020), Chitradevi *et al.* (2021) and Shankar *et al.* (2022). Rapid growth stress in broilers at 4 to 6 weeks age might increase the susceptibility to the infection at grower stage. Desi birds were also affected around 2-7 weeks age, suggesting increased susceptibility in younger birds. This aligns with previous findings reported by Mete *et al.* (2021). Their study highlighted FAdV C-4 as a potential cause of IBH in adult backyard hens with high mortality but without classical gross lesions or hydropericardium commonly associated with HHS. In comparison to broilers, desi birds exhibited a lower incidence of IBH infection, suggesting that their slower growth rates may reduce their susceptibility to the disease. Layers with gizzard erosions were suspected for IBH, but these farms were tested negative

for FAdV. However, Chitradevi *et al.* (2020) detected FAdV in layer birds with gizzard erosions. The absence of PCR positivity in the current study could be attributed to the involvement of other non-adenoviral pathogens or non-infectious causes in the observed gizzard erosions.

The gross post mortem lesions observed in the suspected birds were enlarged, pale and friable liver, accompanied by necrotic foci and ecchymotic haemorrhages in some cases. The liver changes observed may be due to FAdV predilection for the liver (Chitradevi *et al.*, 2021), where it induces hepatocellular damage. This damage leads to inflammation, necrosis and haemorrhages, causing the liver to appear pale, enlarged, friable and were in agreement with reports of Palanivelu *et al.* (2014) and Chitradevi *et al.* (2021).

The gross lesions, like a flabby heart with varying degrees of hydropericardium, characterized by the accumulation of straw-coloured fluid in the pericardial sac observed in this study, were consistent with the lesions reported by Zhao *et al.* (2015) and Chitradevi *et al.* (2021) in broilers. The kidneys of the suspected birds were found to be congested, swollen and showing haemorrhagic patches on their surfaces. Consistent with the present findings, Chitradevi *et al.* (2021) reported similar changes in the kidneys of IBH suspected broilers.

Sequences obtained were subjected to NCBI blast analysis, which revealed the five sequences from field samples exhibited 99% similarity with serotype FAdV 11 sequences. This was in agreement with Gulhane *et al.* (2016), Chitradevi *et al.* (2021), and Shankar *et al.* (2022). Phylogenetic analysis based on partial hexon gene sequences is an effective method for the differentiation and genotyping of FAdV (Islam *et al.*, 2023). Phylogenetic tree revealed that all five field sequences were closely clustered with FAdV-11 of species D sequences from Brazil, Canada, USA and different states of India, including Telangana, Tamil Nadu and Rajasthan. Occurrence of FAdV-11 in IBH are consistent with the reports of Chitradevi *et al.* (2021) and Shankar *et al.* (2022), who also identified FAdV-11 as a prevalent strain in field samples. These findings indicated homogeneity of strains responsible for outbreaks in this region.

Widespread use of monovalent serotype 4-based vaccines to control IBH in this area might have decreased FAdV-4 from the field. This clearly shows that there is no cross protection among different serotypes of FAdV, because serotype 11 is prevalent

in this region. Previous research also opined lack of cross-protection between serotypes (Hess, 2020; Shankar *et al.*, 2022) which emphasizes the need for multivalent vaccines targeting the prevalent strains. However, in some districts, outbreaks were recorded in broiler flocks despite using multivalent vaccines (4,8 and 11), this may be due to vaccination failure or neutralization of vaccine candidates by maternal antibodies.

The multiple sequence alignment of the partial hexon gene nucleotide sequences revealed that all five selected field sequences exhibited 99% and 100% homology with FAdV-11 sequences of foreign and Indian origins, respectively. These findings align with Chitradevi *et al.* (2021), who reported nucleotide homology ranging from 98.6% to 100%. The slight difference in homology between Indian and foreign FAdV-11 sequences could be attributed to geographical variations.

The present study concludes that FAdV serotype 11 of species D was responsible for IBH outbreaks in broiler flocks of Chittoor, Guntur, NTR, Palnadu and Prakasam districts of Andhra Pradesh. Therefore, the development of a multivalent vaccine, including the FAdV serotype 11, may be warranted to prevent IBH disease in poultry within this geographic region of Andhra Pradesh.

Conflict of interest: No conflict of interest in this study.

Data availability statement: This study forms part of the author's research work conducted as part of their M.V.Sc. thesis, which was submitted to Sri Venkateswara Veterinary University (SVVU), Tirupati. The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Author's contribution: MS: Involved in conceptualization and study design; BD: Engaged in data curation, supervision and final editing; TSR: Involved in supervision of the study; KBN: Conducted the research work for this study.

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