RESEARCH ARTICLE

Molecular Detection and Isolation of Fowl Adneovirus Serotype 11 from Chicken Flocks in Kerala

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Abstract

Fowl adenovirus (FAdV) is prevalent across the globe, leading to significant economic setbacks in poultry sector. FAdV infection predominantly affect broilers, also there have been sporadic accounts involving commercial layers. The present research work was attributed to molecular detection and isolation of FAdV from poultry flocks in Kerala. Tissue samples of liver, kidney, heart were collected from flocks showing symptoms and lesions suspecting for FAdV infection. DNA was extracted from these clinical specimens by Phenol-chloroform method. PCR for the detection of FAdV targeting hypervariable region of L1 loop of hexon gene was carried out. Out of 50 samples tested, 5 broiler flocks of age 3 weeks were found positive for FAdV, based on the amplification of partial hexon gene. The hexon gene sequence of these isolates showed that the FAdVs isolates were belonging to serotype 11 of FAdVD genotype group on BLAST analysis. Virus isolation was performed in specific pathogen free (SPF) eggs and in chicken embryo fibroblast (CEF). The inoculated embryos showed symptoms of curling and dwarfing. Haemorrhages and necrosis were observed in liver of the infected embryos. The infected cells in CEF showed cytopathic effects, characterized by swelling, rounding, and detachment. Further, the isolate in the embryonated chicken eggs and CEF cell culture were confirmed by PCR.

Key words: FAdV Serotype 11, Fowl adeno virus, Hexon gene, Inclusion body hepatitis.

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Introduction

The poultry farming domain is more dynamic and plays significant role in global food sector. Also, it serves as an essential component in meeting the nutritional requirement of populations worldwide (Hafez and Attia, 2020). Nevertheless, various infectious diseases pose a persistent threat to this sector, which has direct impact on poultry health, production, and economic stability. Fowl adeno virus stand out among various pathogens causing serious threat to poultry industry (Shah *et al.*, 2011). Fowl adenovirus, a double-stranded DNA virus without an envelope, falls under the Aviadenovirus genus within the Adenoviridae family. Classification is based on molecular structure into five different species (A to E), while 12 serotypes (FAdV-1 to 8a and 8b to 11) are identified through cross-neutralization assays (Cui *et al.*, 2020). Understanding the epidemiology and pathogenesis of infections caused by FAdV requires an understanding of serotyping, as different serotypes exhibit differing degrees of virulence and clinical signs (Steer *et al.*, 2015).

Inclusion body hepatitis (IBH) is one of the most prevalent, primarily affecting broiler chickens of age group between three to four weeks and leading to substantial liver damage, high mortality rates and significant financial losses for poultry farmers (Dutta *et al.*, 2017). IBH which mostly affects broiler chickens and can result in severe liver damage and high death rates, is one of the most common symptoms. IBH outbreaks can result in significant financial losses in poultry industry. Apart from IBH, other conditions that FAdV can cause include egg drop syndrome, quail bronchitis, pancreatic erosions, gizzard erosions (GE), turkey haemorrhagic enteritis, cardiovascular disorder, marble spleen disease, hematopoietic, and respiratory system disorders (El-Shall *et al.*, 2022). Clinical and pathological manifestations of Fowl adeno virus (FAdV) infections present in various forms. The virus spreads horizontally among birds within a flock through the oral-fecal route, with subsequent mechanical transmission facilitated by fecal contamination. Additionally, commercial egg hatching may serve as a mechanism for the spread of the disease between countries (Kataria *et al.*, 2013).

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In various parts of India, the prevalence of IBH, HHS has been well-documented by Karnam *et al.* (2022). However, in Kerala, there hasn't been comprehensive study of this particular virus. Therefore, this study was conducted to detect FAdVs isolates circulating in poultry flocks of Kerala, so as to help in adopting effective control measures and management practices against the disease

Materials and Methods

A total of 50 dead and ailing chicken with the symptoms and lesions suggestive of FAdV infection formed the study material. Tissue samples of liver, kidneys, heart and lungs were collected from dead birds, which were brought to Department of Veterinary Pathology, College of Veterinary and Animal Sciences, Pookode (Kerala, India) for post-mortem. Cloacal swabs were also collected from flocks showing symptoms suggestive of FAdV infection from different poultry farms in Kerala. The clinical specimens were collected and stored at -80°C until use.

Molecular Detection of FAdV: DNA Extraction and Amplification of FAdV's Hexon Gene

The genomic DNA of the virus was extracted from each sample using conventional phenol: chloroform method as described by Green and Sambrook (2017). The extracted DNA was further used as template for PCR for the detecting FAdV targeting hypervariable region of L1 loop of hexon gene. Followed by DNA isolation, the forward primer 5'- CBGCBTRCA TGTACTGGTA- 3' and the reverse primer 5'- AATGTCACNACCGARAAGGC-3' were used as described by Niczyporuk (2018). The optimised reaction condition was performed with initial denaturation for 95°C for 5 min followed by 37 cycles each of denaturation of 95°C for 30 sec, annealing temperature was 55 °C for 45 sec, extension temperature was 72°C for 1 min, and final extension temperature was 72°C for 5 min. After the reaction, the PCR products were detected by 1 % agarose gel electrophoresis.

Sequencing and BLAST Analysis of Hexon Gene of FAdV

The positive PCR products were sent to Genespec Private limited, Kochi, India for sequencing. With the help of Chromas Lite 2.01 software, the chromatograms of the sequences were analysed and trimmed for analysis. To confirm the presence of gene specific to FAdV, BLAST was conducted, the hexon gene sequences of FAdV isolates from Kerala were compared to those of other Indian and foreign FAdV isolates archived in the GenBank as described by Chitradevi *et al.* (2021).

Isolation of the Virus in Embryonated Chicken Eggs (ECE)

Isolation of the virus was performed on 10-day-old Specific pathogen free (SPF) embryonated chicken eggs (ECE) via allantoic route (Safwat *et al.*, 2022). 0.2 mL of filtered positive tissue homogenates were inoculated into Specific pathogen free (SPF) eggs. The uninoculated eggs were kept as control. All the eggs were incubated at 37.5 °C, further eggs which survived after 24 h were harvested. The embryos underwent chilling before being harvested the next day. The harvested embryos were then examined for characteristic lesions associated with FAdV, with comparisons made to control eggs, and PCR was employed again to confirm the presence of FAdV in the harvested embryonic tissues.

Preparation and Isolation of Virus in Chicken Embryo Fibroblast (CEF)

Chicken embryo fibroblasts (CEF) were cultured using 11-day-old Specific pathogen free (SPF) chicken embryos following standard protocols (Abghour *et al.,* 2019). After complete CEF monolayer culture formation, maintenance media was added along with 100 U/mL penicillin and 0.1 mg/mL gentamicin. Around 100 µL of inoculum as described earlier was introduced into the culture flask. Both infected as well as uninfected cell control flasks were kept in the incubator at 37°C with 5% CO_2, and the monolayers were monitored daily for cytopathic effects (CPE) over a period of 5 days. After the completion of CPE in the infected flasks, cells were freezed and thawed for 3 times, later cells were centrifuged at 28 g for 10 min. Subsequently, 100 µL of the supernatant was used to inoculate freshly prepared CEF monolayers as described by Abghour *et al.* (2019).

Results and Discussion

50 dead and ailing birds with symptoms suggestive for FAdVs were screened for FAdV targeting hexon gene in PCR. During necropsy investigation, characteristic lesions in particular straw-coloured fluid in pericardial sac was observed, and liver was friable and pale (Fig. 1). Similar observations were noticed by Sawale *et al.* (2012). The gradient PCR of DNA extracted showed better annealing temperature at 55 °C and the amplicon size obtained was 897 bp. From the 50 samples screened, 5 samples were found positive for FAdV by PCR (Fig 2). Molecular techniques like PCR are frequently utilized for virus detection due to their sensitivity and quick diagnostic capabilities (Asthana *et al.*, 2011). The FAdV genome contains numerous proteins with diverse functions. One key structural protein is hexon, which plays a crucial role, possessing a neutralizing epitope. It has been demonstrated to be serotype-specific, making hexon gene sequencing the primary method for FAdV serotyping (Elbestawy *et al.*, 2020). Studies focusing on hexon gene sequences has identified a highly variable region (HVR) consisting of segments L1, L2, L3, and L4, which contain antigenic and immunogenic elements. These segments are primarily utilized in typing and distinguishing different strains (Chitradevi *et al.,* 2018).

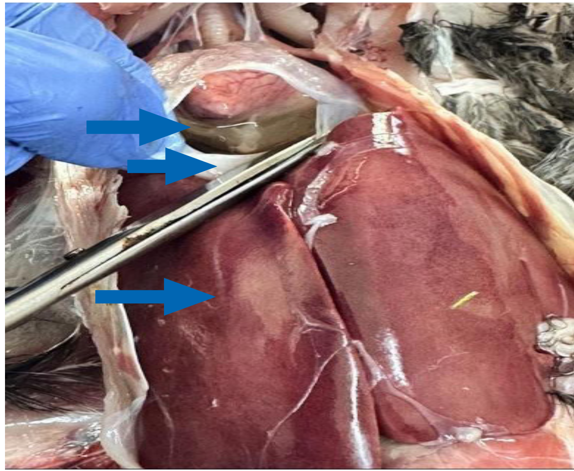

Fig. 1: Accumulation of straw-coloured fluid in pericardial sac, liver was pale and friable.

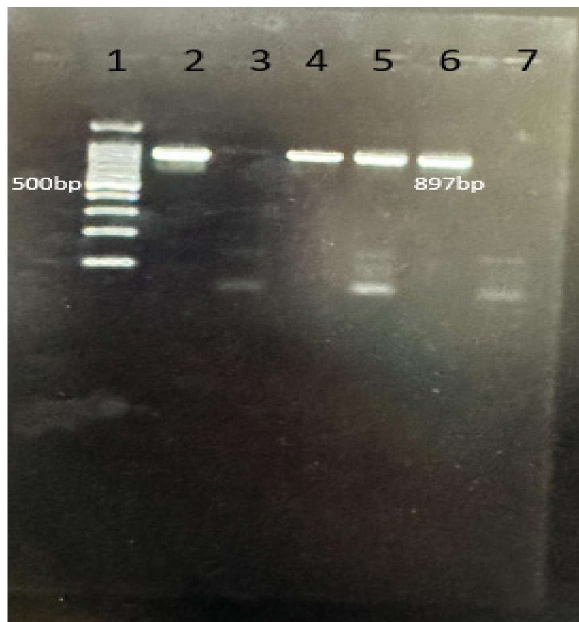

Fig. 2: PCR Amplification of *hexon* gene of FAdV. From left, lane 1: 100bp marker, lane 2: Positive control, Lane 4-6: PCR product *hexon* gene- 897 bp and lane 7: Negative control

Isolating and identifying a virus through virus isolation and identification procedures stands as the definitive method for confirming the virus presence, also it is recognized as the gold standard in diagnostic protocols. FAdV isolation can be conducted in both cell culture and in Embryonated chicken eggs (ECE). According to Abghour *et al.* (2019), the ECE method has been recognized as a sensitive medium for isolating FAdV. In the present study, noticeable differences were observed in the gross appearance of the inoculated embryos compared to the controls (Fig. 4). The embryo showed symptoms of curling, dwarfing and the liver lesion of inoculated embryo was necrotic and haemorrhagic. Every sample that yielded positive amplicons through PCR also tested positive for virus isolation. Similar findings were reported by Gulhane *et al.* (2016). The acquired sequences (Accession no. OQ732022) underwent BLAST analysis and were compared to reference sequences (Accession no. MN540444, MN428137) from both Indian and foreign isolates (Fig. 5), as accessible in the GenBank, which revealed the sequence identity of the positive samples varying from 99.63 % to 99.38 % with Indian and foreign isolates. The genotype analysis of hexon gene confirmed that all the isolates of FAdVs from chicken were related to serotype 11 of D genotype group. Abghour *et al.* (2019) and Chitradevi *et al.* (2021) reported similar results in Morocco and Tamil Nadu.

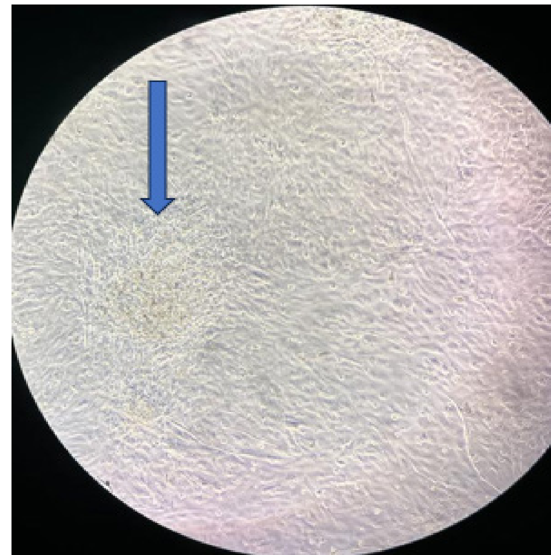

Fig. 3a: Cytopathic effect in 2nd passage of chicken embryo fibroblast-Rounding and clumping, detachment was observed

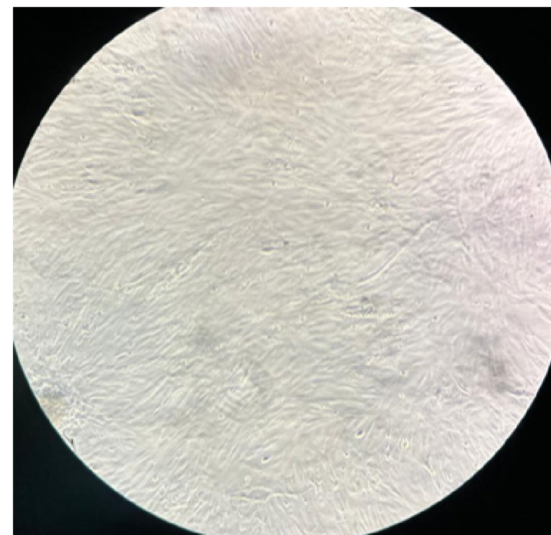

Fig. 3b: Negative control- chicken embryo fibroblast

The CPE including rounding, clumping and detachment of cells was noticed in first passage after 72 h of inoculation, CPE was noticed within 48 h after second passage (Fig. 3 a, b). Similar outcome was obtained by Abghour *et al.* (2019). FAdV isolation is commonly conducted using various cell cultures, with most studies utilizing primary culture such as

chicken embryo liver (CEL) cells and CEF cell culture for this purpose (Kumar *et al.*, 2011). Studies conducted by Mase *et al.* (2012) and Dar *et al.* (2012) reported that FAdV isolation can be attempted in Chicken embryo kidney cell culture as well as in chicken hepatoma cell line.

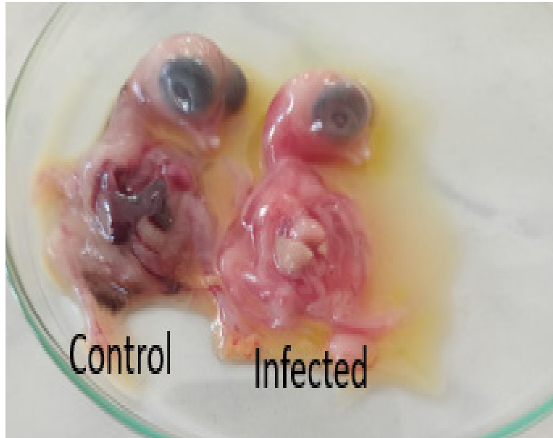

Fig. 4: The infected embryo in embryonated chicken eggs (ECE) showed symptoms of curling, dwarfing, liver appeared necrotic and haemorrhagic.

Conclusion

According to the results of this study, it can be concluded that FAdV serotype 11 is prevalent in broiler chickens aged 3 weeks and older in Kerala. PCR can be considered as an effective method for diagnosing FAdV infection. Isolation and identification of FAdV was successfully performed in SPF embryonated eggs via Allantoic route and CEF cell culture. The study results can be used as guidelines for stakeholders including poultry vaccine manufacturers in India and the epidemiological surveillance units of animal husbandry departments in Kerala and India. Moreover, upcoming research on FAdV should prioritize examining the cross-protective capabilities of various FAdV serotypes, exploring the correlation between immunosuppressive occurrences in FAdV, also investigating diverse vaccination approaches for mitigating IBH and HHS, and developing an effective vaccine.

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blast.ncbi.nlm.nih.gov/Blast.cgi

Sequences producing significant alignments — Download — Select columns — Show 100

select all *100 sequences selected* — GenBank — Graphics — Distance tree of results — MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Fowl aviadenovirus D strain Indovax_FadvD_KHR_17 hexon gene, partial cds	Fowl aviadenovirus D	1458	1458	100%	0.0	99.38%	872	MN540444.1
Fowl aviadenovirus D strain Indovax_FADV_mhr_17 hexon gene, partial cds	Fowl aviadenovirus D	1458	1458	100%	0.0	99.38%	878	MN537891.1
Fowl aviadenovirus D isolate Indovax_FADVD_AJ17 hexon gene, partial cds	Fowl aviadenovirus D	1458	1458	100%	0.0	99.38%	819	MN447717.1
Fowl adenovirus isolate PKFAd18, complete genome	Fowl adenovirus	1458	1458	100%	0.0	99.38%	43840	MN428137.1
Fowl aviadenovirus D isolate FAdV-SAC115 hexon gene, complete cds	Fowl aviadenovirus D	1458	1458	100%	0.0	99.38%	2853	MK995484.1
Fowl aviadenovirus D strain 08-18926, complete genome	Fowl aviadenovirus D	1458	1458	100%	0.0	99.38%	43310	MK572871.1
Fowl aviadenovirus D isolate Ind/TE/16 hexon gene, partial cds	Fowl aviadenovirus D	1458	1458	100%	0.0	99.38%	830	MK816398.1
Fowl aviadenovirus D isolate Pantnagar/HA-16/R-48 hexon protein gene, partial cds	Fowl aviadenovirus D	1458	1458	100%	0.0	99.38%	900	MH379254.1
Fowl aviadenovirus D isolate Pantnagar/HA-16/R-45 hexon protein gene, partial cds	Fowl aviadenovirus D	1458	1458	100%	0.0	99.38%	900	MH379252.1
Fowl aviadenovirus D isolate Pantnagar/KH-14/R-27 hexon protein gene, partial cds	Fowl aviadenovirus D	1458	1458	100%	0.0	99.38%	900	MH379246.1
Fowl aviadenovirus D isolate Pantnagar/KH-14/R-25 hexon protein gene, partial cds	Fowl aviadenovirus D	1458	1458	100%	0.0	99.38%	900	MH379244.1
Fowl aviadenovirus D isolate Pantnagar/PU-14/R-23 hexon protein gene, partial cds	Fowl aviadenovirus D	1458	1458	100%	0.0	99.38%	900	MH379242.1
Fowl adenovirus isolate PANTNAGAR/KA-11/R-8 hexon protein gene, partial cds	Fowl adenovirus	1458	1458	100%	0.0	99.38%	900	MH015228.1
Fowl aviadenovirus D isolate IBH11/chicken/VHB/01/23, partial genome	Fowl aviadenovirus D	1458	1458	100%	0.0	99.38%	43832	OR855393.1
Fowl aviadenovirus D isolate 41/CVAS/PKD/23/KERALA hexon protein gene, partial cds	Fowl aviadenovirus D	1458	1458	100%	0.0	99.38%	804	OR047923.1
Fowl aviadenovirus D strain FADV/F2/23/RS Pura hexon protein gene, partial cds	Fowl aviadenovirus D	1458	1458	100%	0.0	99.38%	880	OR690111.1
Fowl aviadenovirus D isolate 07/08 Palani/TN/Ind hexon protein gene, partial cds	Fowl aviadenovirus D	1458	1458	100%	0.0	99.38%	852	OR684953.1
Fowl aviadenovirus D isolate FAdV-CK-Danish/Pak-OP22-23 hexon protein gene, partial cds	Fowl aviadenovirus D	1458	1458	100%	0.0	99.38%	880	OR644364.1
Fowl aviadenovirus D isolate FAdV-CK-Danish/Pak-OP21-23 hexon protein gene, partial cds	Fowl aviadenovirus D	1458	1458	100%	0.0	99.38%	889	OR472558.1
Fowl aviadenovirus D isolate FADV/F1/22/Kathua hexon protein gene, partial cds	Fowl aviadenovirus D	1458	1458	100%	0.0	99.38%	900	OR371950.1

Fig. 5: BLAST analysis of sequence in comparison to reference sequences accessible in GenBAnk

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