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Molecular Epidemiology of Fowl Adenovirus (FAdV) from Inclusion Body Hepatitis (IBH) Incidences from Indian Broilers Revealed the Prevalence of Serotypes of FAdV-D and E

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ABSTRACT

Background: Inclusion body hepatitis (IBH) caused by Fowl adeno virus (FAdV), is a re-emerging threat to broilers causing considerable economic loss to poultry industry worldwide.

Methods: The present study investigated the IBH outbreaks in commercial broiler flocks from different regions of India between 2019- and 2021 and identified the serotypes associated with it by molecular characterization of hexon gene of FAdV strains.

Result: 42 out of 98 flocks affected with IBH tested FAdV positive by PCR. Phylogenetic analysis grouped these strains into three serotypes; 25 strains (67.56%) were clustered with FAdV-11 serotype, 11 strains (29.72%) with FAdV-8b serotype and one strain with FAdV-8a (2.70%). Three serotypes FAdV-11, 8a and 8b of species D and E are currently circulating in Indian broilers. The present study indicates the need for continuous surveillance of FAdV and provides useful information for devising vaccination strategies for effective control of IBH in chickens.

Key words: Fowl adeno virus (FAdV), Hexon gene, IBH, Molecular serotyping, Phylogenetics.

INTRODUCTION

Fowl adenoviruses (FAdVs) are implicated in wide range of diseases in poultry including inclusion body hepatitis (IBH), hydropericardium syndrome (HPS), gizzard erosions (GE), respiratory and enteric conditions causing severe economic loss worldwide (Adair and Fitzgerald, 2008). FAdVs are double stranded DNA viruses and belongs to the genus *Aviadenovirus* of *Adenoviridae* family. As per the current International Committee on Taxonomy of Viruses (ICTV) classification, there are five species of Aviadenoviruses (A-E) and are further subdivided into 12 serotypes (FAdV-1 to 8a, 8b, 9-11) based on their restriction enzyme digestion pattern and cross-neutralization tests (Meulemans *et al*., 2004).

FAdV serotypes vary in their pathogenicity and are associated with different disease conditions. Hydropericardium syndrome (HPS), popularly known as Angara or litchi disease is primarily associated with FAdV-4 serotype (Choi *et al*., 2012), whereas gizzard erosions (GE) and inclusion body hepatitis (IBH) are mainly associated with FAdV-2, 8a, 8b and 11 serotypes (Mittal *et al*., 2014). The temporal and geographical distribution of different serotypes also varies (Kiss *et al*., 2021). Molecular serotyping based on the hexon coding sequence is widely used in recent times replacing the conventional cross-neutralization assays and restriction fragment length polymorphism assays owing to the latter's tediousness and complexity.

All the serotypes are reported in Indian poultry associated with IBH-HPS with a predominance of FAdV-4 and 8 serotypes. Inactivated FAdV-4 vaccines are used widely in broiler parents in recent years to pass on the passive immunity to commercial broiler chicks. However, outbreaks are often reported from commercial broiler flocks (Shinde *et al*., 2020). Continuous molecular surveillance for identifying the circulating serotypes and serotype specific vaccination is needed for effective control of the disease and its spread. Hence, in the present study we investigated that the serotypes associated with IBH currently circulating in broilers.

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MATERIALS AND METHODS

Sample collection

Tissue samples were collected from clinically affected and IBH suspected commercial broiler flocks from different states of India between April 2019 and June 2021. A total of 98

flocks covering all four zones (North, South, East and West) of India were included in the study. All the birds were reared under deep litter system in open houses. Broilers varying in the age group of 2 and 5 weeks from flocks experiencing 5-30% mortality were sampled. The samples were analysed at ICAR-Directorate of Poultry Research, Hyderabad during 2020-21.

Histopathological examination

The liver tissues collected in 10% neutral formal saline were processed for tissue sections and slides were prepared following standard protocol. The tissue sections were stained by Hematoxylin and Eosin (HandE) stain and visualized under light microscope.

Genomic DNA extraction

DNA was isolated from all tissue samples from 98 flocks by following standard procedure. Briefly, the samples (50 mg) were lysed in lysis buffer containing proteinase K at 37°C for 30 min. DNA was extracted with phenol-chloroform-isoamyl alcohol (25:24:1) twice, followed by precipitation of DNA with absolute isopropanol, washed with 70% ethanol, dried at room temperature and resuspended in nuclease-free water. The extracted DNA was stored at -20°C until further use in PCR.

PCR screening of samples for FAdV by PCR

The PCR was performed with the hexon gene specific primers as described by Meulemans *et al*. (2001) with slight modifications. Briefly, the reaction was carried out in 25 µl reaction using 2 µl of template DNA with 2.5 µl of 10 × Taq buffer with $MgCl_2$, 0.5 µl of each primer (20 pmol), 0.5 µl of dNTPs (10 mMol) and 0.25 µl of Taq DNA polymerase (5 U/ml) and nuclease free water. The PCR conditions were as follows: 95°C for 10 min, 35 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min and final elongation at 72°C for 10 min. The presence of 897 bp product after amplification in the samples were considered FAdV positive and further processed for sequencing.

Sequencing and phylogenetic analysis

The amplified PCR products (hexon L1, 897 bp) from 37 positive flocks were sequenced in both directions using ABI automated sequencer. The obtained sequences were edited and aligned in EditSeq and MegAlign programme of DNA Star software. The nucleotide sequences of field FAdV strains were submitted in NCBI database (Table 1).

A total of 23 reference hexon gene sequences representing all 12 FAdV serotypes were retrieved for analysis (Table 2). The reference sequences along with 37 sequences of the present study were aligned by using Clustal W method and annotated with MEGA X version 10.0.5 (www.megasoftwae.net) (Kumar *et al*., 2018). The best- fit model for analysis was selected by comparing the Bayesian information criterion (BIC) scores. Phylogenetic tree was constructed by using the hexon gene sequences of both reference and current strains by following the Neighbour-joining method with 1000 bootstrap replicates based on Tamura-Nei model (Tamura *et al*., 2011). All positions containing gaps and missing data were eliminated. The FAdV strains were serotyped based on the clustering of the sequences with reference strains in the phylogenetic tree.

Screening for other immunosuppressive viruses by PCR (MD, ALV, REV and CAV)

The DNA samples were used for screening other immunosuppressive viruses by PCR. MD, ALV and REV in the samples were screened by multiplex PCR using primer sets to target genes developed by our lab as described earlier (Kannaki *et al*., 2021). The samples were screened for CAV in a separate PCR using the primers targeting the VP3 region as described earlier (Yilmaz *et al*., 2001).

RESULTS AND DISCUSSION

FAdVs have slowly emerged as primary pathogen associated with IBH, HPS and GE conditions in young broilers causing major economic threat worldwide in recent decade (Kiss *et al*., 2021). In India also, increasing mortality and poor growth rate were observed along with hepatitis outbreaks in young broilers in recent times (Shinde *et al*., 2020). Mortality rate ranged from 5 to 30% in the affected farms. Necropsy findings revealed enlarged pale, friable, yellow coloured liver and haemorrhages on liver in most of the farms. Hydropericardium was not observed in affected farms. Histopathological observation revealed the presence of basophilic intranuclear inclusion bodies in hepatocytes, vacuolation, degeneration and necrosis of hepatocytes, engorgement of sinusoids and haemorrhages in liver. Only two flocks showed gizzard erosions and unthriftiness.

Hexon is the major surface protein of adenoviruses containing the neutralizing epitopes. Hexon gene comprises of conserved pedestal regions P1 and P2 and variable loop regions L1, L2 and L4 (Toogood *et al*., 1989). Hence, gene sequencing of loop 1 of hexon is routinely used for FAdVs serotyping in recent times. 42 out of 98 flocks screened by PCR were positive by hexon gene specific PCR. Remaining 56 samples were negative by PCR.

The phylogenetic analysis based on the hexon loop-1 gene nucleotide sequences of FAdV strains revealed the presence of three serotypes (11, 8b and 8a) in the flocks with IBH (Fig 1). FAdV-11 was the most prevalent 67.56% (25/37) serotype followed by FAdV-8b 29.72% (11/37). The current FAdV-11 strains closely clustered with earlier reported Indian FAdV-11 serotypes (EU847633 and AY581276) from North India. FAdV-8a was observed in only one flock 2.70% (1/37). It clustered with TR59 and 8565 strains of 8a serotype from Belgium and USA respectively. These serotypes belong to D (FAdV-11) and E (FAdV-8a and FAdV-8b) FAdV species respectively.

FAdV-4, 8a, 8b and 11 are considered as potentially virulent serotypes causing severe clinical disease conditions. FAdV 2 and 11 were most widely associated with IBH in broiler with recent increasing incidences associated with 8a and 8b also (Kajan *et al*., 2013). Recently, serotypes

8a, 8b and 11 were also implicated in enteric disease and malabsorption in broilers (Torre *et al*., 2018). Other serotypes cause disease or mortality in the presence of other immunosuppressive agents or co-factors.

At least three species of FAdVs were known to cause clinical disease in many countries including India. Among them, serotypes belonging to D and E species are the most prevalent in many countries. In the present study, FADV-D species is the most prevalent associated with IBH indicating the geographical variation. While the pathogenicity of FAdV-4 in causing HPS is well established with experimental infection studies (Zhao *et al*., 2015), pathogenicity of the other serotypes is scanty (Steer *et al*., 2015) and yet to be established.

The samples tested negative for other immunosuppressive viral pathogens *viz*., MD, ALV, REV and CAV upon screening by PCR. In the present study, we did not observe any HPS condition in affected flocks either alone or with hepatitis. Further sequencing and phylogenetic analysis also shown the absence of FAdV-4 serotype in the outbreaks. Extensive vaccination followed in Indian broiler breeder flocks with inactivated FAdV-4 serotype with targeted passive immunity in broiler chicks may be implicated in significant control of HPS in field conditions and supported by the evidence that FAdV-8b and 11 serotypes become prevalent followed by drastic decline in FAdV-4 cases in South Korea after the introduction of FAdV-4 vaccines (Lai *et al*., 2021). In addition, FAdV-8b has emerged as the dominant

Table 1: Genbank accessions of FAdV field strains of this study.

Sample ID	State	zone	Accession no.	Serotype	Species
FAdV/HYD/21/023/hexon	Karnataka	South	MZ547671	11	D
FAdV/HYD/21/024/hexon	Karnataka	South	MZ547672	11	D
FAdV/HYD/21/032/hexon	West Bengal	East	MZ695028	8b	E
FAdV/HYD/21/033/hexon	West Bengal	East	MZ695029	8b	E
FAdV/HYD/21/034/hexon	West Bengal	East	MZ695030	8b	E
FAdV/HYD/21/035/hexon	West Bengal	East	MZ695031	8b	E
FAdV/HYD/21/036/hexon	West Bengal	East	MZ695032	8b	E
FAdV/HYD/21/037/hexon	Maharashtra	West	MZ836218	11	D
FAdV/HYD/21/038/hexon	Maharashtra	West	MZ836219	11	D
FAdV/HYD/21/039/hexon	Telangana	South	MZ836220	11	D
FAdV/HYD/21/040/hexon	Maharashtra	West	MZ836221	11	D
FAdV/HYD/21/041/hexon	Telangana	South	MZ836239	11	D
FAdV/HYD/21/042/hexon	Telangana	South	MZ836213	8b	E
FAdV/HYD/21/043/hexon	Telangana	South	MZ836222	11	D
FAdV/HYD/21/044/hexon	Telangana	South	MZ836223	11	D
FAdV/HYD/21/045/hexon	Telangana	South	MZ836214	8b	E
FAdV/HYD/21/046/hexon	Telangana	South	MZ836215	8b	E
FAdV/HYD/21/047/hexon	Telangana	South	MZ836224	11	D
FAdV/HYD/21/048/hexon	Telangana	South	MZ836216	8b	E
FAdV/HYD/21/049/hexon	Maharashtra	West	MZ836225	11	D
FAdV/HYD/21/050/hexon	Maharashtra	West	MZ836226	11	D
FAdV/HYD/21/051/hexon	Telangana	South	MZ836227	11	D
FAdV/HYD/21/053/hexon	Telangana	South	MZ836238	8a	E
FAdV/HYD/21/054/hexon	Telangana	South	MZ836228	11	D
FAdV/HYD/21/055/hexon	Telangana	South	MZ836240	11	D
FAdV/HYD/21/057/hexon	Telangana	South	MZ836241	11	D
FAdV/HYD/21/058/hexon	Uttar Pradesh	North	MZ836229	11	D
FAdV/HYD/21/061/hexon	Tamil Nadu	South	MZ836230	11	D
FAdV/HYD/21/062/hexon	Tamil Nadu	South	MZ836231	11	D
FAdV/HYD/21/063/hexon	Telangana	South	MZ836232	11	D
FAdV/HYD/21/064/hexon	Telangana	South	MZ836233	11	D
FAdV/HYD/21/065/hexon	Telangana	South	MZ836234	11	D
FAdV/HYD/21/066/hexon	Karnataka	South	MZ836235	11	D
FAdV/HYD/21/069/hexon	West Bengal	East	MZ869852	8b	E
FAdV/HYD/21/071/hexon	West Bengal	East	MZ836236	11	D
FAdV/HYD/21/075/hexon	West Bengal	East	MZ836217	8b	E
FAdV/HYD/21/077/hexon	West Bengal	East	MZ836237	11	D

serotype recently with initial high prevalence of FAdV-11 for two years followed by the introduction of 4 serotype specific vaccine in China and South Korea also (Wang *et al*., 2018; Lai *et al*., 2021). Emerging 8b strains from different parts of world causes IBH and showed broad tissue tropism with less mortality (Huang *et al*., 2019). Similar trend is observed in the current study too.

Co-infection of FAdVs and other pathogens are often quite common and reported regularly (Brown *et al*., 2019). However, FAdVs have emerged as primary pathogens in causing clinical diseases in recent times. Absence of any co-infection with other immunosuppressive viruses such as MD, ALV, REV and CAV in the present study reiterates the fact that co-infection or presence of other viruses may not

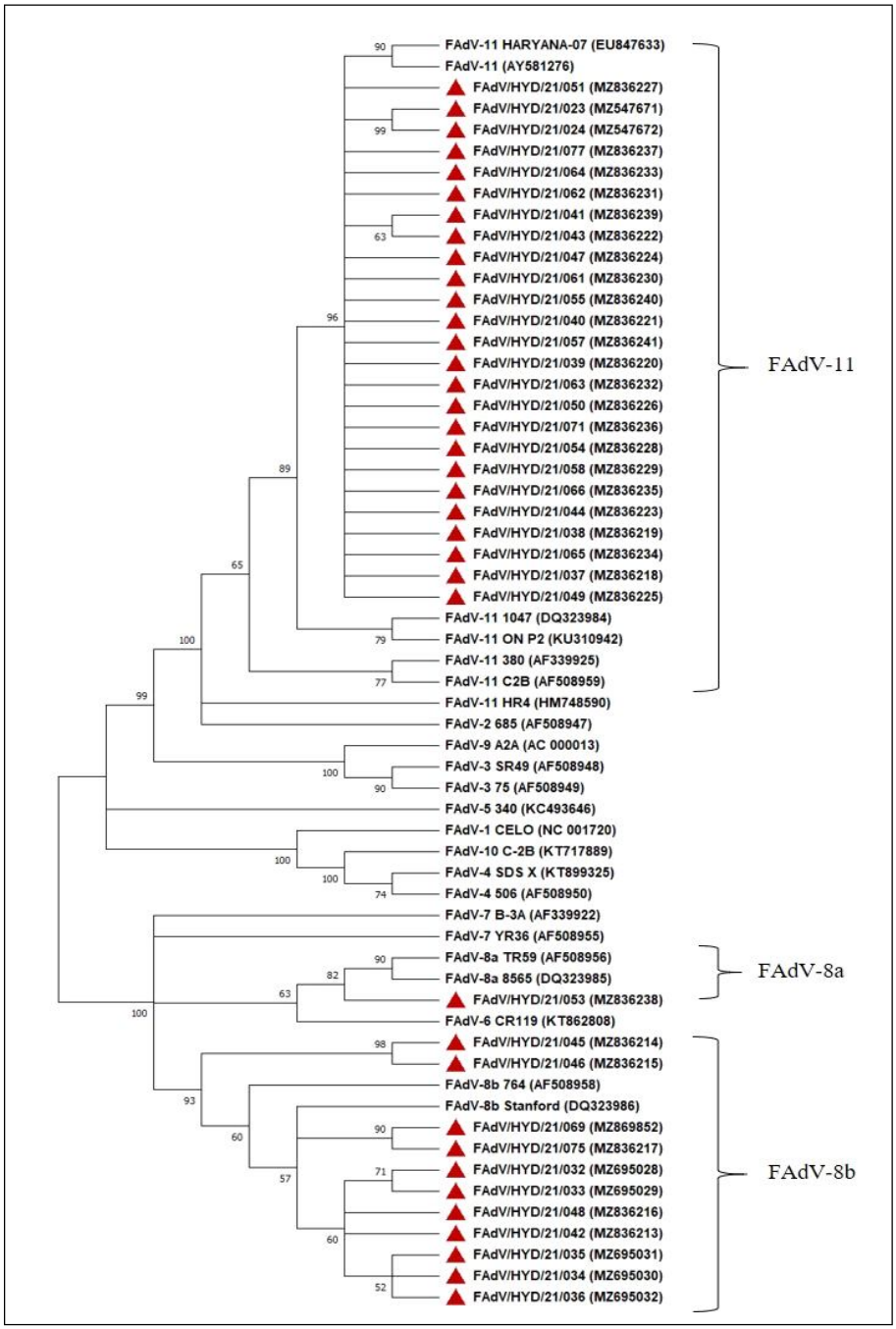

Fig 1: Phylogenetic tree of hexon gene nucleotide sequences of 37 FAdV field strains from this study with 23 reference strains retrieved from NCBI database. Phylogenetic tree was constructed by Neighbour-joining method with 1000 bootstrap replicates based on Tamura-Nei model in MEGA 10.0.5 software. Strains from this study are highlighted with red triangle (Δ).

Table 2: Fowl adenovirus (FAdV) reference strains used in the present study.

Species	Serotype	Strain	Country	Accession number
A	FAdV-1	CELO	Austria	U46933
B	FAdV-5	340	Austria	KC493646
C	FAdV-4	506	Belgium	AF508950
	FAdV-4	SDSX	China	KT899325
	FAdV-10	C-2B	Austria	KT717889
D	FAdV-2	685	Belgium	AF508947
	FAdV-3	SR49	Belgium	AF508948
	FAdV-3	75	Belgium	AF508949
	FAdV-9	A-2A	UK	AC_000013
	FAdV-11	380	Belgium	AF339925
	FAdV-11	1047	USA	DQ323984
	FAdV-11	ABT/AD83/Haryana-07	India	EU847633
	FAdV-11	17/AD/02/Chicken/India	India	AY581276
	FAdV-11	C2B	Belgium	AF508959
	FAdV-11	ON P2	Canada	KU310942
	FAdV-11	HR4	India	HM748590
E	FAdV-6	CR119	Austria	KT862808
	FAdV-7	YR36	Belgium	AF508955
	FAdV-7	B-3A	Belgium	AF339922
	FAdV-8a	TR59	Belgium	AF508956
	FAdV-8a	8565	USA	DQ323985
	FAdV-8b	764	Belgium	AF508958
	FAdV-8b	Stanford	USA	DQ323986

be critical or necessary for these serotypes (FAdV-11, 8b and 8a) in causing the clinical disease conditions as reported earlier by other workers (Steer *et al*., 2011). Challenge studies with these serotypes in SPF chicken have been shown to induce disease earlier.

Almost all the serotypes were reported from India associated with IBH-HPS from different parts of the country. Nevertheless, FAdV-4 and -8 were reported to be prevalent in commercial broilers. Identification of other serotypes in the current study, along with a recent report of FAdV-11 serotype in broilers (Shinde *et al*., 2020) indicates the dynamics of disease landscape. Little or no cross-protection was observed among the serotypes and protection unlikely to occur unless the serotypes belong to same FAdV species (Hess, 2020). Although a previous report showed cross-protection induced by inactivated FAdV-4 vaccine against 8b and 11 in an experimental study (Kim *et al*., 2014), under field conditions similar protection is not observed (Lai *et al*., 2021). Moreover, poor cross-protectivity between 8b and 11 FAdV serotypes was also reported. This reiterates the current observation and emphasis the use of multiple serotypes for vaccination for complete protection.

CONCLUSION

In summary, at least three serotypes of FAdV species D and E were circulating in Indian broilers with high prevalence of FAdV-11 serotype during 2020-2021. Continuous molecular surveillance for the FAdV serotypes and pathogenicity studies are further warranted. The current information would be useful while devising the biosecurity measures and vaccination strategy for effective IBH control.

Conflict of interest

All authors declare that they have no conflict of interest.

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