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Short Paper

Isolation and molecular characterization of fowl adenovirus from inclusion body hepatitis cases in Western India during 2019-2021

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Abstract

Background: Inclusion body hepatitis (IBH) resulted in a substantial economic loss in Western India during 2019 to 2021. **Aims:** The study aimed to characterize fowl adenovirus (FAdV) from field outbreaks. **Methods:** The study was conducted on 290 liver samples from 66 poultry flocks. The samples were subjected to histopathology and molecular detection, followed by phylogenetic typing of the partial *hexon* gene of the virus. **Results:** Spiking mortality (14%) was recorded from day 21 to day 35 with peak mortality at the 28th day of age. The necropsy showed a pale and enlarged liver with hemorrhagic and yellowish necrotic foci, accumulation of straw-colored transudate in the pericardial sac which resulted in a flabby appearance of the heart, heart enlargement, and hemorrhages on the spleen, enlarged and congested kidneys. The virus inoculation resulted in stunting and poor feathering with hepatomegaly, hemorrhages and yellowish necrotic foci on the liver as well as greenish discoloration, and kidney swelling in SPF embryonated chicken eggs. Out of 29, 16 liver samples yielded 1219 bp amplicons specific to *hexon* gene fragments. The sequence and phylogenetic analysis identified 14 isolates as FAdV species E serotype 11 and two as species D serotype 8b. **Conclusion:** The results indicated that FAdV-8b and FAdV-11 strains are involved in disease outbreaks in western India.

Key words: Adenovirus, Inclusion body hepatitis, India, Isolation, Phylogeny

Introduction

In India, the poultry sector is not only a commercially developed industry but also supports rural livelihood. Commercial broiler farms with small to medium flock sizes are widely distributed in rural India. Many farmers rear indigenous and improved poultry varieties. These flocks often suffer from disease outbreaks leading to economic losses of the farmers. Certain disease outbreaks are observed in vaccinated flocks and in the flocks that are derived from vaccinated ones.

Inclusion-body hepatitis (IBH) is an economically important disease that can cause substantial economic loss to the poultry sector, due to reduced productivity, immune suppression, and mortality (Dahiya *et al*., 2002; McFerran and Adair, 2008). In India, the disease was first reported in 1994 (Gowda *et al*., 1994). Since then, it has caused several outbreaks in commercial poultry flocks throughout peninsular India (Kumar *et al*., 2010). Though the disease is controlled by maternal antibodies in the chicks due to vaccinating the parents, the number of outbreaks have increased in the country during the past few years. The disease has become a major cause of mortality in commercial poultry.

The disease is caused by fowl adenoviruses (FAdVs) and serotypes 2, 8a, and 8b of the virus are isolated from the birds who suffered from IBH (Kumar *et al*., 2010; Schachner *et al*., 2018). The serotype 4 is isolated from the birds who suffered from hydropericardium syndrome (Dahiya *et al*., 2002). These viruses belong to the genus Aviadenovirus of the Adenoviridae family (Niczyporuk *et al*., 2021). The FAdVs have 12 serotypes divided into five different species from A to E (Hess, 2013). The broiler chickens of the age group of 3-5 weeks are most susceptible to the disease (Asthana *et al*., 2013) which results in rapid mortality ranging from 10 to 60% of the

flock. Recently, Chitradevi *et al*. (2021) characterized FAdV serotype 11 from the IBH field outbreaks in different Indian states. However, all the 12 serotypes can cause the disease. The literature reported outbreaks from north and south India however; substantive data from western India is lacking. Therefore, the etiology needs to be characterized. Moreover; though the FAdV serotype 11 was suggested to be involved in the recent IBH outbreaks (Chitradevi *et al*., 2021), the role of other serotypes in the current outbreaks cannot be ruled out.

Therefore, this study was conducted to characterize the IBH outbreaks in western India by studying the symptoms, necropsy, and histopathological features, followed by isolation of the etiological agent and its molecular identification.

Materials and Methods

Sampling

Broiler (n=37) and village poultry flocks (n=29) were visited in western India from 2019 to 2021. The liver samples (n=290) were collected from 29 poultry flocks of age groups between 15 to 35 days, where mortality was suspected to be due to IBH. The flock size varied between 1000 to 6000 birds. The sick birds showed lethargy, dullness, prostration, huddling, ruffled feathers, inappetence, sulfur yellow colored diarrhea, and body weight loss with sudden mortality (Figs. 1a, b and c) and overall 14% spiking mortality (Fig. 2) ranging from 3.3 (native chicken) to 55.28% (broiler chicken) in different broiler flocks was recorded.

The carcasses were subjected to post-mortem examination and the samples were collected from 29 flocks suspected to be involved with IBH. Samples from liver, pancreas, and kidney (one from each flock) were collected in 10% formal saline for histopathology. The liver samples were collected aseptically in a virus transport medium and were transported to the laboratory by maintaining a cold chain.

Sample processing

The tissue samples (29 liver, 29 pancreases, and 29 kidneys) were processed for histological investigations. The tissue sections (4 µm thick) were stained with haematoxylin and eosin following standard protocols.

The liver samples collected in the virus transport medium were homogenized in phosphate buffer saline (pH 7.2) to obtain 10% suspension. These suspensions were freeze-thawed three times, clarified by centrifugation, filtered through 0.22 µm membrane filters, and stored at -80°C until further use.

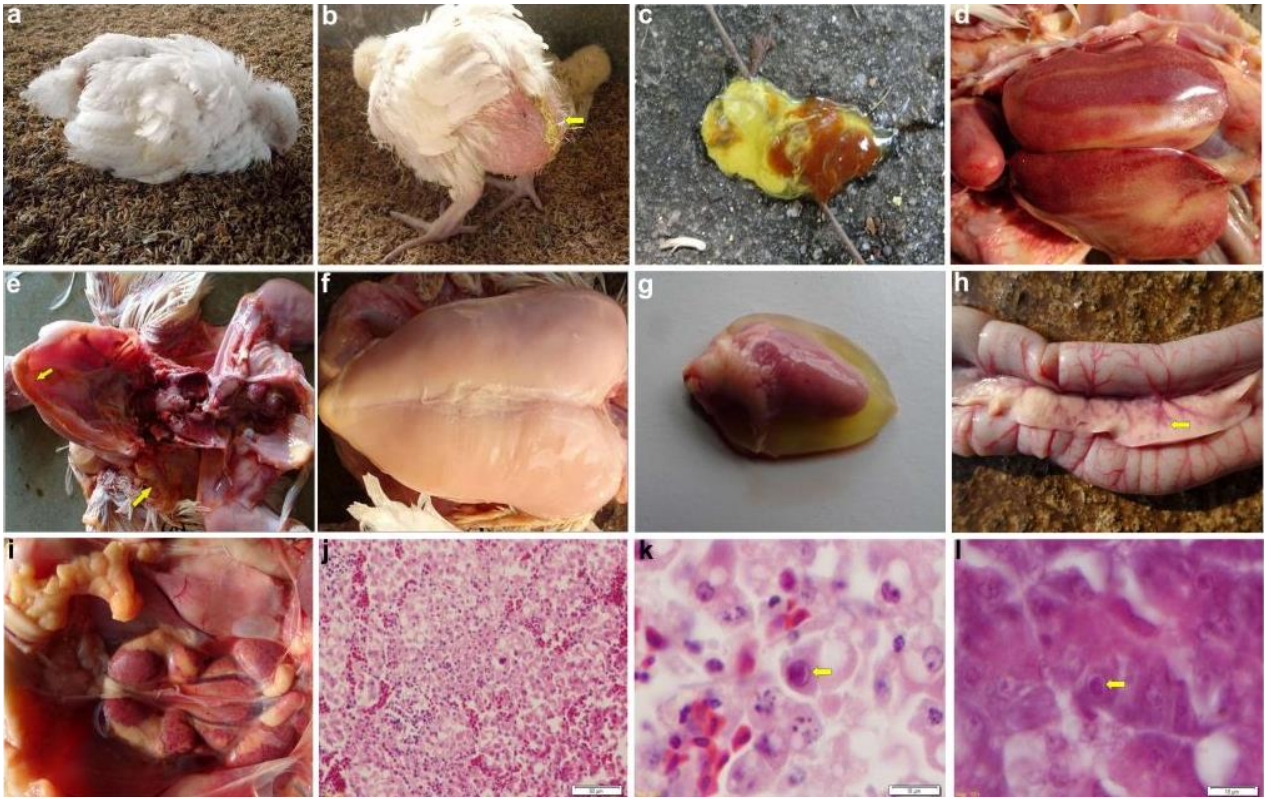

Fig. 1: Necropsy and histopathological findings. (**a**, **b** and **c**) Dullness, prostration, ruffled feathers and sulphur yellow colored diarrhea, (**d**) Pale icteric markedly swollen or enlarged and mottled livers with reticular pattern due to presence of widespread petechial to ecchymotic hemorrhages, (**e**) Icteric discoloration of subcutaneous tissue or fat (arrows), (**f**) Pallor appearance of breast musculature, (**g**) Hydropericardium, (**h**) Pancreatic swelling and presence of multiple necrotic foci, (**i**) Enlarged, pale kidneys with multiple hemorrhages, (**j** and **k**) Multifocal to locally extensive hepatocellular degeneration and necrosis, congestion and hemorrhages, mononuclear cell infiltration and presence of basophilic intra-nuclear inclusion bodies (arrow), (H&E staining, scalr bar: j=50 µm, and k=10 µm), and (**l**) Exocrine pancreatic acinar cells degeneration and necrosis, focal mononuclear cell infiltration and presence of basophilic intranuclear inclusions (arrows), (H&E staining, scale bar, 10 µm)

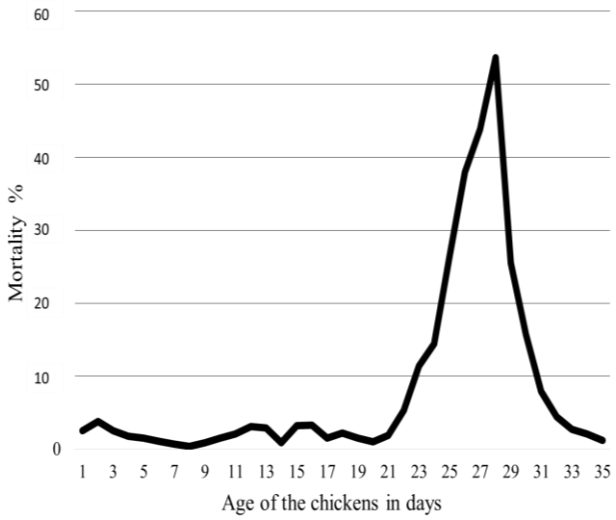

Fig. 2: Mortality pattern. Flock spiking mortality pattern in IBH infected chicken flocks

Virus isolation

The isolation of FAdV was performed in 12-day-old embryonated specific pathogen free (SPF) eggs by chorioallantoic (CAM) route. The eggs with healthy embryos were swabbed with 70% alcohol. The inoculum (100 µL) was dropped on the inner shell membrane in an artificial air sac with the help of a 1 ml tuberculin syringe. The holes were sealed with melted wax. The eggs were incubated at 37°C in an incubator with 50 55% humidity for 10 days. The eggs were candled twice daily. Deaths before 24 h of inoculation, if any, were omitted. The virus replication was confirmed by cytopathic effects (CPE) and histopathology of two representative embryonic liver.

Identification

The liver tissues were subjected to DNA extraction followed by PCR targeting the amplification of the partial hexon gene (L1 and L2 region). The PCR amplification of the partial *hexon* gene was carried out at the annealing temperature of 49°C for 1 min using (H1) 5´TGG GAC ATG GGG GCG ACC TA3´ and (H2) 5´AAG GGA TTG ACG TTG TCC A3´ primers (Raue and Hess, 1998). The PCR products were electrophoresed on 1% agarose gel and visualized in a gel documentation system (Biorad, USA).

Sequence analysis

The nucleotide sequences were analyzed using BLASTn (http://blast.ncbi.nlm.nih.gov) and aligned in ClustalW (http://www.megasoftware.net/).

Phylogenetic analysis

The aligned sequences were subjected to phylogenetic analysis by the Neighbor-Joining method in MEGA X software (http://www.megasoftware.net/) using 1000 bootstrap repetitions based on the Poisson correction model and Kimura 2-parameter model (Kimura *et al*., 1980; Tamura *et al*., 2011). The representative nucleotide sequences of five FAdV

Table 1: Details of the fowl adenovirus strains used in the phylogenetic analysis

SN	Accession No.	Strain	Country	Year	Species	Serotype
1	OK491822	FAdV_UD1/1	India	2019	D	FAdV-11
2	OK491823	FAdV_UD4/1	India	2019	D	FAdV-11
3	OK491824	FAdV_UD7/1	India	2020	D	FAdV-11
4	OK491825	FAdV_UD9/1	India	2020	D	FAdV-11
5	OK491826	FAdV_UD12/1	India	2020	D	FAdV-11
6	OK491827	FAdV_ML13/1	India	2020	D	FAdV-11
7	OK491828	FAdV_ML14/1	India	2020	D	FAdV-11
8	OK491829	FAdV_KR15/1	India	2020	D	FAdV-11
9	OK491830	FAdV_JK18/1	India	2020	D	FAdV-11
10	OK491831	FAdV_RD19/1	India	2020	D	FAdV-11
11	OK491832	FAdV_RD20/1	India	2020	D	FAdV-11
12	OK491833	FAdV_RD21/1	India	2020	D	FAdV-11
13	OK491834	FAdV_MK22/1	India	2020	D	FAdV-11
14	OK491835	FAdV_MK24/1	India	2020	D	FAdV-11
15	OK491836	FAdV_MK23/1	India	2020	E	FAdV-8b
16	OK491837	FAdV_MK23/2	India	2020	E	FAdV-8b
17	AC_000014	-	UK	2003	A	FAdV-1
18	MK757473	LYG	China	2017	B	FAdV-5
19	HQ709228	Kr-Yeoju	Korea	2010	C	FAdV-4
20	KT717889	C-2B	USA	2015	C	FAdV-10
21	KT862805	685	UK	1960	D	FAdV-2
22	KT862807	SR49	Japan	1960	D	FAdV-3
23	NC_000899	-	Canada	2020	D	FAdV-9
24	MK995485	FAdV-SAC126	S. Arabia	2018	D	FAdV-11
25	NC_038332	CR119	Japan	1960	E	FAdV-6
26	KY364398	SD15-21	China	2015	E	FAdV-7
27	MF198256	SDRZ	China	2011	E	FAdV-8a
28	MK572865	14-259	France	2014	E	FAdV-8b
29	MH349775	Duck	China	2015	-	FAdV-3

species and 12 serotypes were retrieved from the GenBank and included in the analysis (Table 1). FAdV-3/Duck (MH349775) sequence was employed as the out group for both of the analyses.

Results

Necropsy findings

The livers of affected birds were found markedly swollen or enlarged, pale icteric with soft fragile texture, and mottled with reticular pattern due to the presence of widespread petechial to ecchymotic hemorrhages (Fig. 1d). The vent feathers were soiled with sulfur yellow colored mucoid droppings. Icteric discoloration of subcutaneous tissue or fat, and pallor appearance of breast musculature were also observed (Figs. 1e and f). Hydropericardium (Fig. 1g), marked pancreatic swelling along with multiple necrotic foci (Fig. 1h), and gizzard erosions were evident in a few affected birds. The kidneys were enlarged and pale, and revealed multiple haemorrhages (Fig. 1i).

Histopathological findings

The liver revealed multifocal to local hepatocellular degeneration and necrosis, congestion, hemorrhages, occasional mononuclear cell infiltration (Fig. 1j), and basophilic intra-nuclear inclusion bodies (Fig. 1k). Mesangial proliferative glomerulitis and marked cortical tubular degeneration and necrosis were observed in kidneys. The pancreas showed multifocal to coalescent areas of exocrine pancreatic acinar cell degeneration and necrosis occasionally associated with focal mononuclear cell infiltration and the presence of basophilic intranuclear inclusions (Fig. 1l).

Virus isolation

The infected embryos showed subcutaneous hemorrhages (Fig. 3a), stunted growth, and poor feathering (Figs. 3b and c). The livers were abnormally enlarged with necrotic foci, greenish discoloration and congestion (Figs. 3d, e and f). The kidneys were swollen and congested (Fig. 3g). Amniotic fluid was clear, but CAM was slightly thick with small hemorrhagic and white spots. The CPE in the second passage was milder compared to the first passage. On histopathology, the liver of the embryos showed mild to moderate degrees of hepatocellular degeneration, congestion or hemorrhages, and the presence of basophilic intra-nuclear inclusion bodies (Figs. 3h and i). The embryos from the negative control group showed normal growth with normal feathering. Amniotic fluid was clear. The internal organs, *viz*. liver, kidney, heart, and CAM, were normal.

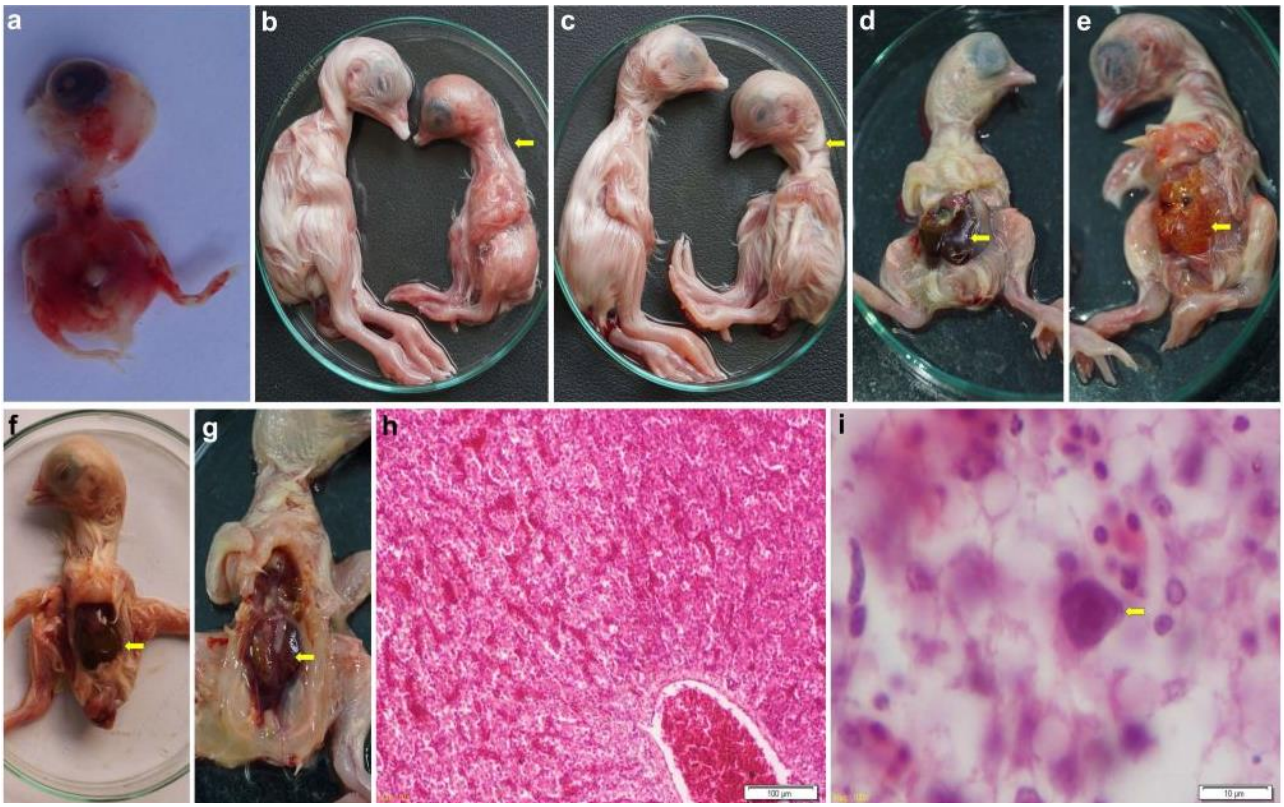

Fig. 3: (**a**) Cytopathic effects of fowl adenovirus in SPF chicken embryos (day 4 post inoculation). Infected embryo showed stunted growth and subcutaneous hemorrhages, (**b** and **c**) Stunted growth and poor feathering, (**d**, **e**, **f** and **g**) Hepatomegaly, hemorrhages and necrotic foci on liver, (**h** and **i**) Liver (chick embryos inoculated with IBH virus). Mild to moderate degree of hepatocellular degeneration, congestion or hemorrhages and presence of basophilic intra-nuclear inclusion bodies (arrow), (H&E staining, scale bar: h=100 µm, and i=10 µm)

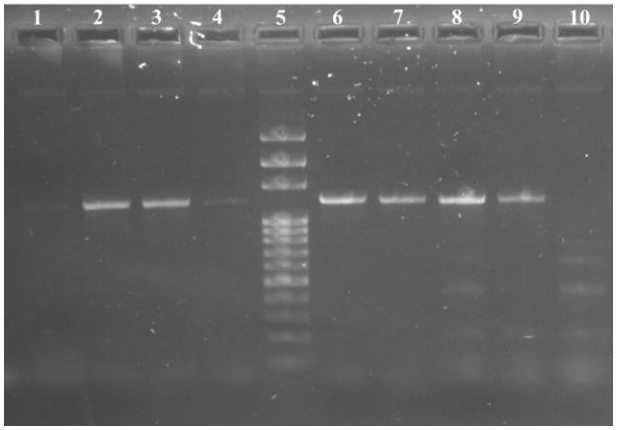

Fig. 4: PCR amplicons of partial *hexon* gene of fowl adenovirus from field samples. Lanes 1-4 and 6-9: 1219 bp amplicons, Lane 5: 100 bp DNA marker, and Lane 10: Negative

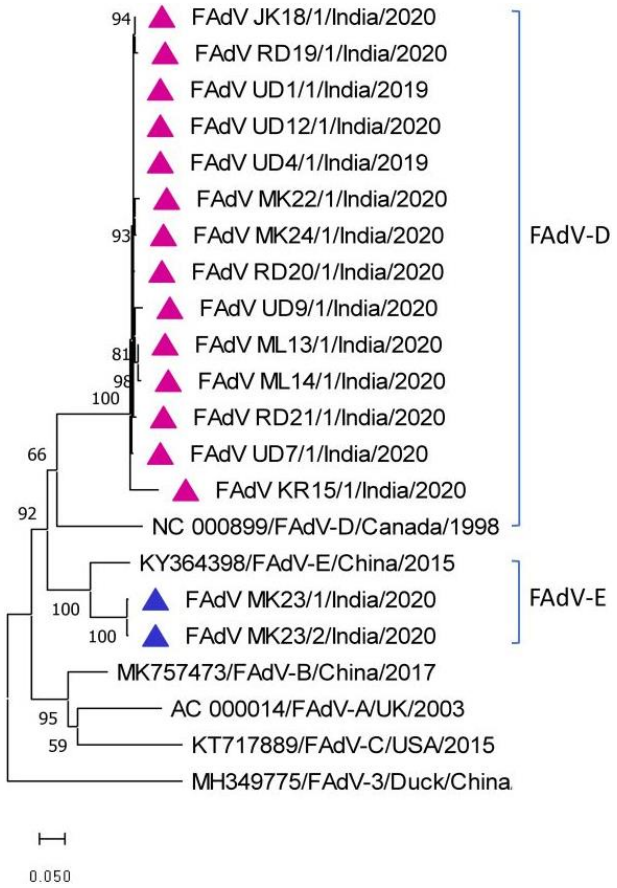

Fig. 5: Species based phylogenetic analysis of the fowl adenovirus strains with the reference GenBank sequences of partial *hexon* gene sequences. FAdV covering the species A to E were included for the analysis. Phylogenetic tree was constructed by the Neighbor-Joining method and bootstrapped for 1000 replications

Amplification and phylogenetic analysis of the partial *hexon* gene

The PCR amplified 1219 bp specific amplicon at an annealing temperature of 49°C (Fig. 4). The nucleotide sequences are deposited in the GenBank with accession numbers from OK491822 to OK491837 (Table 1). In species-wise phylogenetic analysis, 14 field FAdV isolates were placed in a cluster along with FAdV species D, while the other two were in another clade along with FAdV species E. All FAdV isolates and reference sequences were clustered in a major cluster and a duck FAdV was placed as an out group (Fig. 5). The serotype-wise phylogenetic analysis clustered the 14 field isolates with FAdV serotype 11, and the other two with FAdV serotype 8b (Fig. 6).

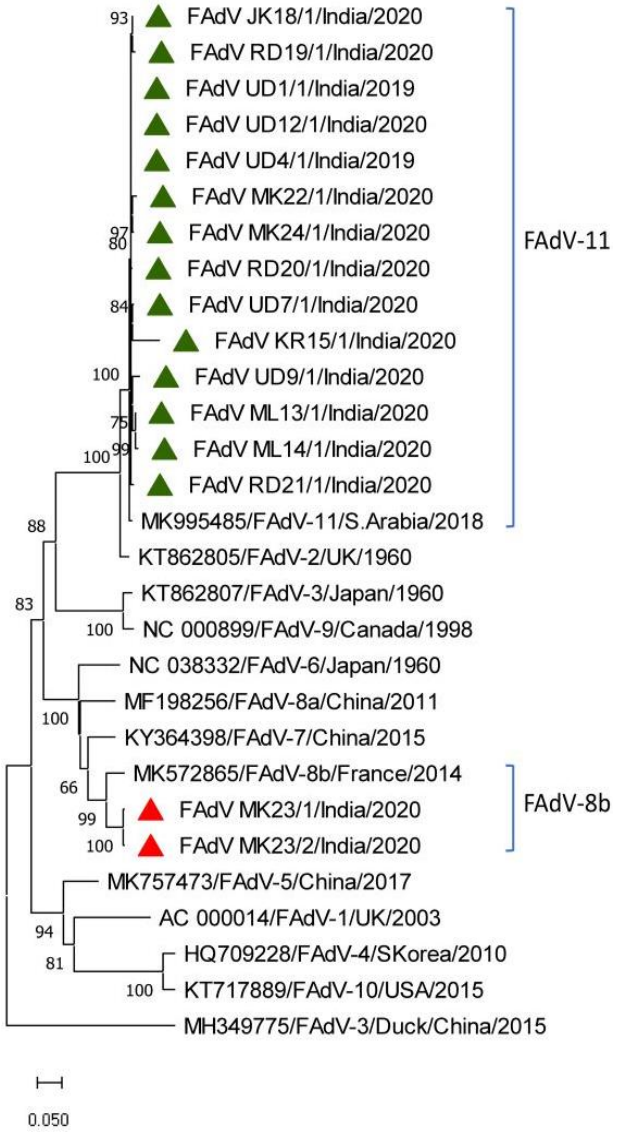

Fig. 6: Serotype based phylogenetic analysis of the fowl adenovirus strains with the reference GenBank sequences of partial hexon gene sequences. FAdV serotype 1 to 11 were included for the analysis. Phylogenetic tree was constructed by the Neighbor-Joining method and bootstrapped for 1000 replications

Discussion

The clinical manifestations of the infections caused by fowl adenoviruses are more usually observed in 2 to 6-week-old commercial broiler chickens. The IBH and

hydropericardium syndrome have been reported in India for the past several decades (Chandra *et al*., 2000). The current investigation reported an overall 14% mortality ranging from 3.3 (native chicken) to 55.28% (broiler chicken) in different broiler flocks. Shrivastava *et al*. (2019) reported 2.78 and 15.85% mortality in Kadaknath and commercial broilers, respectively. During our study, a sudden increase in mortality was recorded which lasted for 15 days. The peak mortality was recorded on the 28th day, followed by a sudden drop. This mortality pattern is often referred to as flock-spiking mortality (Merck, 2020).

The adenovirus-infected birds often show lethargy, ruffled feathers, shaking, huddling together, resting on chest and beak depression as well as sulfur yellow colored diarrhea. The abrupt mortality with similar signs was earlier reported in the literature (Dahiya *et al*., 2002; Zhao *et al*., 2016). The infected birds were reluctant to drink water, eat feed, and move as earlier reported (Chandra *et al*., 2000).

The deceased birds showed icteric subcutaneous tissue, pallor appearance of breast musculature, swollen livers, pancreas and kidneys, hydro pericardium, and gizzard erosions. The livers were pale icteric with crumbly fragile texture, and mottled with reticular pattern due to the presence of widespread petechial to ecchymotic hemorrhages. These findings were typical of the disease (Dahiya *et al*., 2002). Zhao *et al*. (2016) reported discolored liver with foci of hemorrhage and/or necrosis, as found in the present investigation. The histopathology revealed basophilic intranuclear inclusions in hepatocytes and pancreatic acinar cells. Similar findings were recorded by Sohaimi *et al*. (2019).

A variety of pathologies like stunting and curling of the embryos, green or necrotic livers, enlarged spleens, congestion and bleeding of body parts, urates in the mesonephros and poor feathering were stated in CAM-inoculated embryos. The CPE of the present study is in accordance with these findings except for the curling of the embryos.

The PCR is one of the widely used, specific, sensitive, and advantageous methods to detect FAdVs in clinical samples. Further, it provides a confirmatory diagnosis (Abghour *et al*., 2019). In the present investigation, PCR could amplify 1219 bp specific *hexon* gene amplicon. This amplification confirmed the replication of FAdV in the affected poultry flocks and inoculated embryos.

The species-wise phylogenetic analysis showed two major clusters. The first cluster consisted of FAdV-A, FAdV-B, and FAdV-C, while another cluster consisted of FAdV-D and FAdV-E. The analysis placed 14 field FAdVs (FAdV UD1/1, FAdV UD4/1, FAdV UD7/1, FAdV UD9/1, FAdV UD12/1, FAdV ML13/1, FAdV ML14/1, FAdV KR15/1, FAdV JK18/1, FAdV RD19/1, FAdV RD20/1, FAdV RD21/1, FAdV MK22/1, and FAdV MK24/1) closely with NC000899 (FAdV-D) isolate. The other two field FAdVs (FAdV MK23/1 and FAdV MK23/2) were closely placed with FAdV-E (KY364398) isolate.

The serotype-wise phylogenetic analysis formed two major clusters. The first cluster consisted of FAdV-1, FAdV-5, FAdV-4, and FAdV-10, corresponding to FAdV-A, FAdV-B, and FAdV-C species. Another major cluster was divided into two clades. The FAdV-6, FAdV-7, FAdV-8a, and FAdV-8b formed separate clade. The FAdV MK23/1 and FAdV MK23/2 were closely placed with FAdV-8b forming distinct sub-clade from FAdV-6, FAdV-7, and FAdV-8a. Another sub-clade was formed by FAdV-2, FAdV-3, FAdV-9, and FAdV-11. FAdV-3 and FAdV-9 were placed distinctly in this sub-clade. The remaining 14 FAdV field strains (FAdV UD1/1, FAdV UD4/1, FAdV UD7/1, FAdV UD9/1, FAdV UD12/1, FAdV ML13/1, FAdV ML14/1, FAdV KR15/1, FAdV JK18/1, FAdV RD19/1, FAdV RD20/1, FAdV RD21/1, FAdV MK22/1, and FAdV MK24/1) were clustered with FAdV-11.

fowl adenoviruses serotypes 2, 8a, 8b, and 11 were isolated from the birds who suffered from IBH (Schachner *et al*., 2018; Niczyporuk *et al*., 2021), and serotype 4 from the birds who suffered from hydropericardium syndrome (Dahiya *et al*., 2002). fowl adenoviruses 2, 5, 6, 7, 8, and 12 were stated to be the cause of IBH outbreaks from India (Mittal *et al*., 2014) and other parts of the world (McFerran and Adair, 2008; Mase *et al*., 2009; Lai *et al*., 2021). A recent report recorded serotype 11 as a predominant cause of IBH in India (Chavan *et al*., 2023).

FAdV-8b and FAdV-11 strains are involved in disease outbreaks in western India.

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Conflict of interest

The authors declare that there are no conflict of interest.

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