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Molecular typing of fowl adenovirus associated with gizzard erosion in commercial layer grower chicken in Tamil Nadu

S CHITRADEVI*, K SUKUMAR, P SURESH, G A BALASUBRAMANIAM and D KANNAN

Tamil Nadu Veterinary and Animal Sciences University, Namakkal, Tamil Nadu 637 002 India

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ABSTRACT

The present study was undertaken to characterize fowl adenovirus associated with commercial layer grower chicken showed gizzard erosion. Ninety four commercial layer grower chicken flocks from Namakkal districts of Tamil Nadu had shown reduced feed intake, reduced weight gain, uneven growth and mortality of 0.3 to 7.7%. On postmortem examination of affected birds showed mild to severe gizzard erosion, blackish discoloration of gizzard contents, pale liver and no major lesions were seen in other organs. Total DNA was extracted and 897 bp fowl adenovirus specific hexon gene was amplified by PCR. Out of 94 flocks screened seven flocks were found positive of fowl adenovirus. Chicken embryo liver cell culture was prepared to isolate field fowl adenovirus from suspected flocks. Concurrent infection of chicken anaemia virus (CAV) was also screened by PCR for 419 bp VP2 gene of CAV and found that all the seven flocks which were PCR positive for FAdV also found positive for CAV. Sequencing and phylogenetic analysis of 897 bp FAdV hexon gene revealed that, it was belonged to FAdV serotypes 2 and 3 of species D.

Keywords: Commercial layer grower chicken, Fowl adenovirus, Gizzard erosions, PCR, Phylogenetic analysis, Sequencing

Fowl adenovirus (FAdV) comes under the family Adenoviridae, genus *Aviadenovirus*. *Aviadenovirus* has been grouped into five species (A to E) based on their molecular structure and further subdivided into 12 serotypes based on their restriction enzyme digestion pattern and serum cross neutralization test (Hess, 2000). The fowl adenovirus has been associated with number of disease condition like Inclusion body hepatitits (IBH), Hydropericardial syndrome (HPS), IBH-HPS, respiratory tract disease, tenosynovitis and gizzard erosion (Adair and Fitzgerald, 2008). In recent days gizzard erosions in commercial broiler and layer birds also cause economic losses to the farmers by the way of uneven growth, reduced feed intake and body weight. Many factors like diets that are deficient in Vitamin B6, ingestion of histamine, toxic substances such as mycotoxin and gizzerosine (Gjevre *et al*. 2013). First time adenoviral gizzard erosions in layer chicks was reported in 1993 (Tanimura *et al*. 1993). Gizzard erosion outbreaks due to FAdV serotype 1 infections were reported in commercial broiler chickens in Japan, Europe and Korea (Ono *et al*. 2001, Marek *et al*. 2010, Grafl *et al*. 2013, Schade *et al*. 2013). Recently in India the report of gizzard erosions in commercial layer grower chicken was reported by Bulbule *et al*. (2016). Involvement of many serotypes of FAdV causing infections, the present study was aimed to identify the serotypes involved in causing gizzard erosion by molecular methods and it is useful to control the disease.

*Corresponding author e-mail: chitradevi.dr@gmail.com

MATERIALS AND METHODS

Sample collection: From October 2016 to September 2018, 9 to 13 weeks old commercial layer grower chicken flocks showed mortality, uneven growth, dullness and reduced feed intake in and around Namakkal district, Tamil Nadu, India. Liver and gizzard samples were collected from 94 commercial layer grower flocks for disease diagnosis and further analysis.

Histopathological examination: After necropsy examination, the liver and gizzard tissues collected from affected chicken were fixed with 10% formalin. The formalin fixed tissues were processed by paraffin wax embedding method for tissue sectioning and were stained with haematoxyline and eosin (H&E) stain (Bancroft and Stevens, 1996). The H&E stained slides were read under microscope and histopathological changes were recorded.

Virus isolation: For virus isolation 10% suspension of pooled liver and gizzard tissue homogenates were prepared with sterile phosphate buffered saline with *p*H 7.2 and freeze thawed three times. The homogenates were centrifuged at 2,000 × g for 10 min, the supernatant was treated with antibiotic and antimycotics (100 units of penicillin G, 100 µg of streptomycin and 0.25 µg of amphotericin B) and kept for 1 h at room temperature. After centrifugation at 2,000 × g for 10 min, the supernatant was filtered through

0.45 µm Millipore membrane filter and used for virus isolation. Primary chicken embryo liver cell (CELi) cultures were prepared from 13 to 15 days old embryonated chicken eggs as per the method described by Barua and Rai (2003) with slight modifications.

DNA extraction and Polymerase chain reaction: The DNA was extracted from pooled liver and gizzard tissues from each flock by using DNA extraction kit (Catalog No.51304, Qiagen, USA). The quantity and purity of DNA was assessed by Nanodrop^{TM} (Thermo scientific, USA). The polymerase chain reaction of fowl adenovirus specific 897 bp hexon gene was carried out as per Meulemans *et al.* (2001) with slight modification in cycle condition. The primer sequence used in this study was: forward 5'CAARTTCAGRCAGACGGT 3' and reverse 5'TAGTGATGMCGSGACATCAT 3'. The PCR was carried out in a final volume of 20 ml containing 10 µl of 2x Red dye master mix (Amplicon, USA) (consisting of 0.05 units/ µL Taq DNA polymerase, 150 mM Tris HCL (*p*H 8.5), 40 mM $(NH_4)_2$ SO_4, 4.0 mM Mg^{2++},0.4 mM of

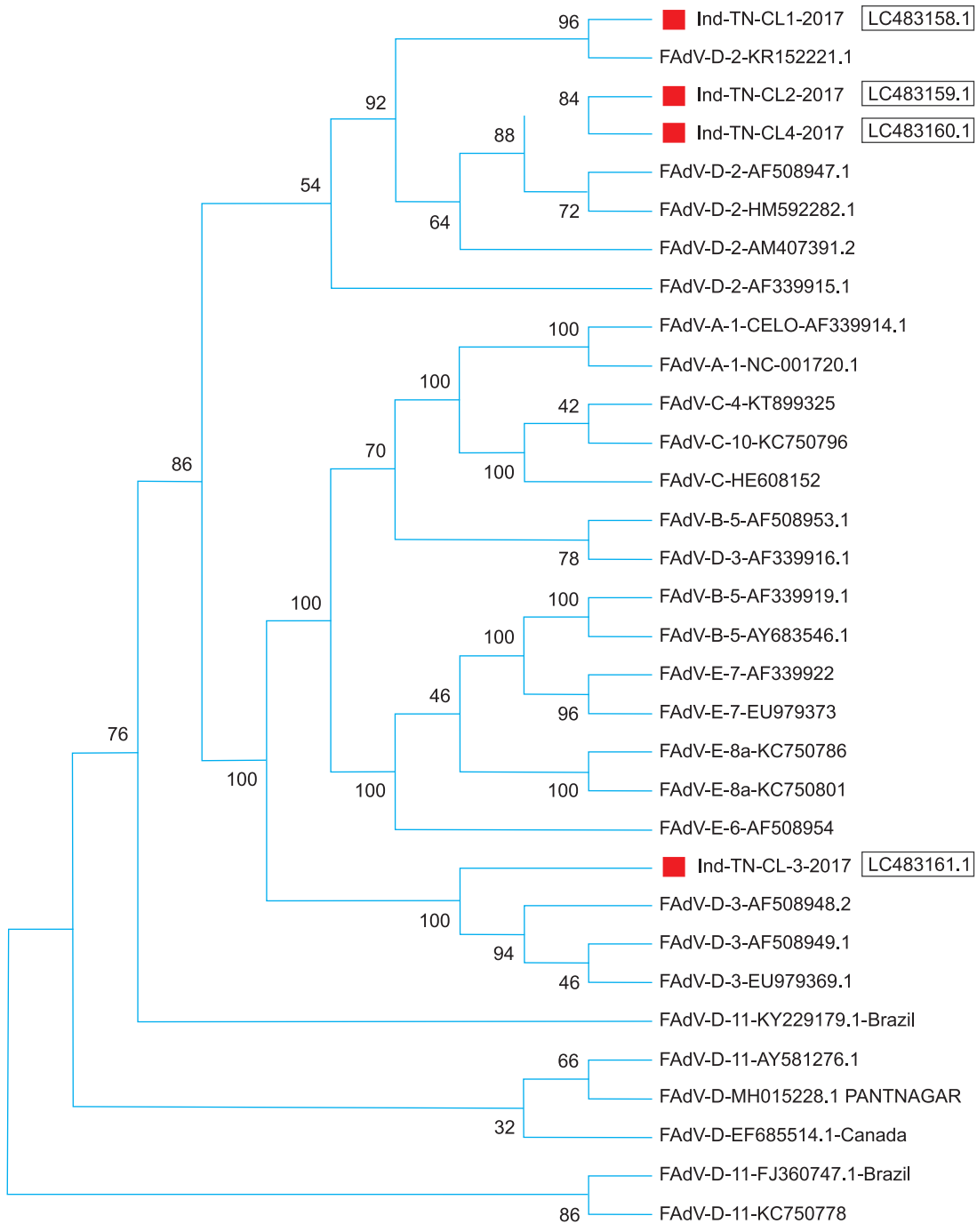

Fig. 1. Phylogenetic analysis of 897 bp L1 hexon gene of field FAdV isolates with other reference sequences from GenBank.

Table 1. Commercial layer grower flock details for fowl adenovirus (FAdV) and Chicken anaemia virus (CAV) confirmation

Farm number	Age (wks)	PCR positives		Farm number	Age (wks)	PCR positives	
		FAdV	CAV			FAdV	CAV
1	12	Negative		48	10	Negative	
2	12	Positive		49	9	Negative	
3	9	Negative		50	10	Negative	
4	11	Negative		51	10	Negative	
5	10	Negative		52	10	Negative	
6	12	Negative		53	10	Negative	
7	9	Negative		54	11	Negative	
8	9	Negative		55	10	Negative	
9	9	Negative		56	8	Negative	
10	10	Negative		57	10	Negative	
11	11	Positive	Positive	58	9	Negative	
12	11	Positive	Positive	59	9	Negative	
13	11	Positive	Positive	60	11	Negative	
14	13	Positive	Positive	61		Negative	
15	9	Positive	Positive	62	11	Negative	
16	10	Positive	Positive	63	12	Negative	
17	9	Negative		64	9	Negative	
18	6	Negative		65	9	Negative	
19	9	Negative		66	10	Negative	
20	12	Positive	Positive	67	11	Negative	
21	11	Negative		68	13	Negative	
22	12	Negative		69	12	Negative	
23	8	Negative		70	11	Negative	
24	11	Negative		71	10	Negative	
25	9	Negative		72	10	Negative	
26	9	Negative		73	11	Negative	
27	9	Negative		74	10	Negative	
28	13	Negative		75	12	Negative	
29	11	Negative		76	11	Negative	
30	12	Negative		77	9	Negative	
31	10	Negative		78	12	Negative	
32	9	Negative		79	10	Negative	
33	14	Negative		80	12	Negative	
34	11	Negative		81	10	Negative	
35	11	Negative		82	9	Negative	
36	10	Negative		83	11	Negative	
37	12	Negative		84	9	Negative	
38	10	Negative		85	11	Negative	
39	9	Negative		86	12	Negative	
40	11	Negative		87	10	Negative	
41	13	Negative		88	10	Negative	
42	7	Negative		89	12	Negative	
43	10	Negative		90	9	Negative	
44	12	Negative		91	11	Negative	
45	8	Negative		92	11	Negative	
46	12	Negative		93	10	Negative	
47	11	Negative		94	11	Negative	

each dNTP, 0.2% Tween 20 and Inert red dye and Stabilizer), each 1 µl of forward and reverse primer (10 pmol/ µl), 3 µl of DNA and 5 µl of nuclease free water. The reaction was carried out in a thermal cycler (Multigene optimax, Labenet, USA) with initial denaturation at 94ºC for 10 min, denaturation at 94ºC for 5 min, annealing at 62ºC for 30 sec, extension at 72ºC for 2 min followed by 35 cycles final extension of 72ºC for 10 min. The agarose gel was prepared with 1.5% agarose containing ethidium bromide 1.5 µl/ 25 ml TAE buffer and amplified 897 bp hexon gene PCR products were visualized UV gel documentation system. Concurrent infection of chicken anaemia virus infection with FAdV was also screened by PCR for 419 bp VP2 gene of CAV as per Ottiger (2010).

Sequencing and phylogenetic analysis: The four purified PCR products were cloned in pTZ57R/T cloning vector and the recombinant clones were confirmed by hexon gene specific PCR and subjected for sequencing. The both

forward and reverse hexon gene nucleotide sequences were aligned using BioEdit version 7.0 sequence alignment editor. Homology searches were conducted using the NCBI program BLAST and FAdV reference serotype sequences were retrieved from the GenBank data base and phylogenetic analysis of the nucleotide sequences of hexon gene was performed with maximum likelihood method with Taimura 3 parameter model using MEGA version 7.0.

RESULTS AND DISCUSSION

Fowl adenoviruses are ubiquitous, relatively stable in the environment and are often isolated from chicken populations. They can cause various pathologies, in nature. The present study indicates an outbreak of fowl adenovirus associated gizzard erosions in commercial layer grower chicken flock. The clinical signs observed in this study were dullness, reduced feed and water intake, reduction in weight gain, uneven growth and mortality of 0.3 to 7.7% between 9 and 13 weeks of age group. On necropsy examination, affected birds showed pale and slightly enlarged liver, mild to severe gizzard erosion and blackish discolouration of gizzard contents. No major gross lesions were observed in lungs, bursa of Fabricius and spleen of FAdV infected birds. Clinical signs and postmortem findings observed in this study was well supported by the findings of Bulbule *et al*. (2016) who recorded FAdV infection in 6 to 13 weeks of commercial layer grower chicken in India. The variation in mortality percentage might be due to age, breed, immune status, viral load during infection, concurrent infection and involvement or variation of serotypes in FAdV infection. On histopathological examination of gizzard sections revealed that the disruption and hyalinization and focal disruption of gizzard muscle fibres and mononuclear cells infiltration. Moderate degenerative changes with foamy cytoplasm and acidophilic intranuclear inclusions in hepatocytes were also noticed. Histopathological gizzard lesions observed in this study were similar to those reported previously in chickens naturally or experimentally infected with FAdV (Ono *et al*. 2001, Okuda *et al*. 2001). For virus isolation with CELi cells showed cytopathic effect (CPE) after third passage. The CPE indicated the presence of vacuole and honey comb appearance in third passage at 24 h post infection followed by cell rounding, clumping, detachment and floating of cells. The FAdV field isolates were well adopted and isolated in CELi cells. Many researchers (Jadhao *et al*. 2003, Soumyalekshmi *et al*. 2014, Trivedi *et al*. 2018) had used CELi cells for isolation of FAdV and observed similar findings as that of our present study.

In the present study, the FAdV associated with gizzard erosions was confirmed by amplification of 897 bp fragment containing the L1 loop of hexon gene. Out of 94 commercial layer grower chicken flocks screened against FAdV, seven flocks from Namakkal district of Tamil Nadu were found positive by PCR for FAdV. The per cent PCR positivity for FAdV field isolates from commercial layer grower chicken was and 7.4% respectively. The similar study was conducted by (Lim *et al*. 2012, Shade *et al*. 2013, Bulbule *et al*. 2016) because the hexon protein is the major surface protein of adenovirus, on which type, group and subgroup specific antigenic determinants were located (Russel, 2009). Hence hexon gene was selected for PCR amplification and detection of FAdV genome. Primer pair Hexon A and Hexon B was able to amplify conserved regions in the two pedestal regions adjacent to loop 1 variable region which enables to amplify all the serotypes of FAdV (Meulemans *et al*. 2001). Choi *et al*. (2012) stated that immunosuppression before or concurrently with FAdV infection served as an important factor for developing clinical presentations. In this study, we could found all the FAdV positive flocks had shown PCR positive for 419 bp VP2 gene of chicken anaemia virus (CAV). Similar findings were reported by Bulbule *et al*. 2016, Niu *et al*. 2017, Chitradevi *et al*. 2018).

Genotyping of FAdV associated with gizzard erosions were carried out by (Choi *et al*. 2012, Mase *et al*. 2014). In our study, four samples were selected and subjected for sequencing and phylogenetic analysis revealed that three isolates (Genbank accession number LC483158.1, LC483159.1, LC483160.1) grouped into serotype 2 (Belgium isolate - F339915, India - KR152221, Austria - AM407391, Belgium - AF508947 and Italy - HM592282) and only one isolate (Genbank accession number LC483161.1) showed close relationship with serotype 3 of FAdV species D of Belgium isolates (Belgium-AF508948) and all these isolates were comes under FAdV species D (Fig 1). This is in agreement with Bulbule *et al*. (2016) who characterized FAdV isolates associated with gizzard erosion (GE) in commercial layer birds in India and phylogenetic analysis of the hexon loop L1 gene revealed the presence of FAdV serotypes 1,4,2,3 and 11. Similarly Niczyporuk *et al*. (2013) confirmed field FAdV isolates from chicken in Poland by PCR specific for hexon gene encoding L1 loop and phylogenetic analysis of sequence revealed that all the isolates belonged to five species (FAdV A-E) and eight serotypes (FAdV 1, 2, 4, 5, 7, 8a, 8b and 11) whereas Xia *et al*. (2017) also studied phylogenetic analysis of hexon loop 1 gene of FAdV isolates from China and found that 4.5% of isolates were grouped into FAdV serotype 2. Based on the sequencing and phylogenetic analysis of FAdV isolated gizzard erosions in commercial layer grower chicken revealed the presence of FAdV serotype 2 and 3.

In conclusion, fowl adenovirus serotype 2 and 3 was involved in causing gizzard erosions in commercial layer grower chicken and the presence of immunosuppressive chicken anaemia virus may aggravate the disease condition.

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